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Dissertação Orientada pelo Prof. Doutor Nuno Taveira e co-orientada pelos Prof. Doutora Patrícia Cavaco Silva e Prof. Doutor José Moniz Pereira

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“Be a virus, see the world”

Gary Larson

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Preface

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The studies described in this thesis were performed at the Unidade de Retrovírus e Infecções Associadas – Centro de Patogénese Molecular da Faculdade de Farmácia de Lisboa. The results obtained were published in manuscripts already published or in preparation:

Bártolo I, Camacho R, Barroso H, Bezerra V e Taveira N. **Rapid clinical progression to AIDS and death in a persistently seronegative HIV-1 infected heterosexual young man.** AIDS. 2009;23(17):2359-2362;

Bártolo I, Casanovas J, Bastos R, Rocha C, Abecasis AB, Folgosa E, Mondlane J, Manuel R e Taveira N. **HIV genetic diversity and transmitted drug resistance in health care settings in Maputo, Mozambique.** J Acquir Immune Defic Syndr. 2009;51(3):323-31;

Bártolo I, Rocha C, Bartolomeu J, Gama A, Fonseca M, Mendes A, Epalanga M, Cristina F, Thamm S, Cavaco Silva P, Taveira N. **Antiretroviral drug resistance surveillance among treatment-naive HIV-1-infected individuals in Angola: evidence for low level of transmitted drug resistance.** Antimicrobial Agents and Chemotherapy. 2009;53(7):3156-3158. Epub 2009 May 11;

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Bártolo I, Rocha C, Bartolomeu J, Gama A, Marcelino R, Fonseca M, Mendes A, Epalanga M, Cavaco Silva P, Taveira N. **Highly divergent subtypes and new recombinant forms prevail in the HIV/AIDS epidemic in Angola: New insights into the origins of the AIDS pandemic**. Infect Genet Evol. 2009;9:672-682. Epub 2008 May 9;

Bártolo I, Abecasis AB, Borrego P, Barroso H, McCutchan F, Camacho R, Taveira N. **Origin and epidemiologic history of HIV-1 CRF14_BG**. (Manuscript accepted for publication in PLoS ONE);

Oliveira V, **Bártolo I**, Borrego P, Rocha C, Valadas E, Barreto J, Almeida E, Antunes F, Taveira N. **Genetic diversity and drug resistance profiles in HIV-1 and HIV-2 infected patients from Cape Verde Islands.** (Manuscript submitted to AIDS Research and Human Retroviruses).

Other Publications

Published manuscripts:

Borrego P, Barroso H, **Bártolo I**, Família C, Quintas A e Taveira N. **Evolutionary and structural features of the C2, V3 and C3 envelope regions underlying the differences in HIV-1 and HIV-2 biology and infection**. PLoS ONE. 2011;6(1):e14548.

Submitted manuscripts:

Santos A, Clemente S, **Bártolo I**, Palladino C, Cavaco Silva P, Franco V, Epalanga M, Pinto R, Taveira N. **Evaluation of the diagnostic performance of the rapid test VIKIA HIV1/2 in a highly complex HIV-1 epidemic.** Diagnostic Microbiology and Infectious Diseases. 2011; (In press);

Borrego P, Calado R, Marcelino JM, **Bártolo I**, Família C, Rocha C, Cavaco-Silva P, Doroana M, Antunes F, Maltez F, Caixas U, Barroso H, Taveira N. **Baseline susceptibility of primary Human Immunodeficiency Virus Type 2 to entry inhibitors.** (Manuscript submitted to Antiviral Therapy).

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Taveira N, Borrego P, **Bártolo I. Biologia Molecular de VIH** - Manual sobre SIDA - 3ª Edição. Editor Francisco Antunes. Permanyer Portugal. 2008; 27-50;

Rocha C, **Bártolo I**, Barroso H, e Taveira N. **New insights into the relationship between HIV-2 evolution and disease progression** - VIII HIV/AIDS Virtual Congress – Current research insights into HIV/AIDS and related diseases. SIDAnet, Associação Lusófona. 2008; 251-262;

Rocha C, Barroso H, **Bártolo I,** Marcelino J, Rosado L e Taveira N. **Evolução molecular do gene env do HIV-2 e colapso do sistema imunitário em doentes infectados por via vertical** - VII HIV/AIDS Virtual Congress – O VIH na Criança e no Idoso. SIDAnet, Associação Lusófona. 2007; 251-262;

Palma C, Pinheiro C, Lobão D, Silva J, Barroso H, **Bártolo I**, Carvalho P, Matoso P, Rocha C, Bezerra V. **HIV Medicine 2006, Versão Portuguesa.** Flying Publisher. 2007.

Resumo

A infecção VIH/SIDA é um grave problema de saúde pública, particularmente na África subsaariana, onde vivem 67% dos infectados. O diagnóstico, a terapêutica e a prevenção da infecção VIH/SIDA dependem do conhecimento aprofundado da sua epidemiologia bem como dos agentes causadores desta infecção. A diversidade genética de VIH-1 pode ter impacto a vários níveis, nomeadamente, no diagnóstico, transmissão, progressão da doença e emergência de resistências aos fármacos antirretrovirais (revisto no Capítulo 1). Com a recente introdução da terapêutica antirretroviral combinada (TARc) nos países em vias de desenvolvimento, o prognóstico dos doentes que têm acesso a estes fármacos melhorou substancialmente, com uma marcada diminuição das taxas de mortalidade e morbilidade. No entanto, há preocupações relativas à baixa eficácia da TARc nestes países, o que pode levar à rápida e descontrolada emergência de variantes resistentes aos fármacos antirretrovirais e à sua subsequente transmissão. Há vários aspectos que podem levar à baixa eficácia da TARc, nomeadamente a monitorização limitada da carga viral nos doentes em terapêutica, o uso não regulado de fármacos antirretrovirais comprados no mercado negro e a falta de sistemas de vigilância de resistências.

Os estudos de epidemiologia molecular e de resistência aos fármacos antirretrovirais na infecção VIH-1 descritos nesta dissertação foram realizados em Angola, Moçambique, Cabo Verde e Portugal. Estes países têm fortes laços históricos, sociais, culturais e económicos. Contudo, a infecção VIH/SIDA afecta estes países de forma muito diferente. Moçambique é um dos países da África subsaariana com maior taxa de prevalência da infecção VIH-1 (11,5%), tendo províncias com prevalências superiores a 25%. Angola tem uma prevalência moderada de 6,5%, sendo Luanda a

província que apresenta a prevalência mais elevada do país (13,6%). Cabo Verde apresenta uma taxa de prevalência muito baixa para um país Africano, inferior a 1%. Em Portugal a prevalência da infecção VIH/SIDA sendo baixa (0,6%) é, no entanto, uma das mais elevadas da Europa Ocidental. Em Angola, Moçambique e Cabo Verde, tal como na maioria dos países Africanos, a transmissão da infecção faz-se sobretudo por via heterossexual enquanto em Portugal os toxicodependentes por via endovenosa correspondem a cerca de 40% dos casos de infecção. Em Portugal, os subtipos B e G e a forma recombinante CRF14_BG dominam a epidemia de infecção por VIH-1 mas pouco se sabe sobre a sua origem e história epidemiológica. Relativamente a Angola, Moçambique e Cabo Verde, há pouca ou nenhuma informação sobre os subtipos virais, sobre a sua origem e história epidemiológica e sobre o seu potencial impacto no diagnóstico, progressão da doença e susceptibilidade e resistência aos fármacos antirretrovirais. Pouco se sabe também sobre a natureza e a prevalência das mutações de resistência (primárias e secundárias). Deste modo, os objectivos desta dissertação foram: 1) caracterizar a diversidade genética de VIH-1 em Portugal, Angola, Moçambique e Cabo Verde, e investigar a origem e história epidemiológica deste vírus nestes países; 2) analisar o impacto da diversidade genética no diagnóstico, progressão para a doença e susceptibilidade à terapêutica antirretroviral; e 3) determinar a prevalência das resistências transmitidas em doentes não tratados dos três países Africanos.

Foram colhidas amostras de plasma de doentes VIH-1 tratados e não tratados, entre 1993 e 2007, de Angola, Moçambique e Cabo Verde (Capítulos 2-5). Para subtipagem e/ou análise de mutações de resistência, foram obtidas sequências dos genes *gag* (p17), *pol* (PR and RT) e/ou *env* (C2C3) utilizando para amplificação um protocolo de PCR desenvolvido no nosso laboratório. Os genótipos virais foram determinados por análise filogenética. As mutações de resistência foram determinadas recorrendo ao *Stanford Genotypic Resistance Interpretation Algorithm*. As mutações associadas com resistência transmitida foram seleccionadas de listas publicadas recentemente. Para definir se uma mutação era um polimorfismo natural de um determinado subtipo, as frequências de todos os polimorfismos encontrados nas sequências de doentes tratados e não tratados, foram comparadas com sequências de todo o mundo do mesmo subtipo e/ou de subtipo B. Nos doentes não tratados infectados com vírus resistentes, o perfil mutacional foi analisado para se poder indicar o regime antirretroviral de 1ª linha mais efectivo. Nos doentes tratados, sempre que possível correlacionou-se a presença de mutações de resistência com parâmetros imunológicos e virológicos e com o regime antirretroviral em uso. Usaram-se métodos filogenéticos para determinar a origem dos vírus resistentes e para determinar se os doentes eram epidemiologicamente relacionados. Na investigação de um caso de infecção VIH-1 seronegativa fez-se uma caracterização inicial de vírus recombinantes CRF14_BG com base em sequências clonais parciais dos genes *gag* e *env* (Capítulo 6). Para a caracterização completa destes CRF14_BGs Portugueses, obtiveram-se sequências genómicas completas a partir de amostras de três doentes, um jovem adulto com infecção seronegativa infectado por via heterossexual e duas crianças infectadas por via vertical, em 1997 (Capítulo 7). A reconstrução da história evolutiva desta CRF foi realizada através de *molecular clock analysis*.

Métodos genéticos e filogenéticos foram usados para determinar o tropismo de um número significativo de isolados CRF14_BG de doentes Portugueses e investigar a selecção positiva na região V3.

Em Angola encontraram-se praticamente todas as formas genéticas de VIH-1 do grupo M excepto o subtipo B (Capítulo 2). Quarenta e sete por cento dos doentes estava infectado com vírus recombinantes, dos quais 36% eram de 2ª geração. Encontraram-se 58 perfis de recombinação diferentes. Encontraram-se ainda numerosos isolados não tipáveis e dois novos grupos de sequências dentro da radiação do subtipo A que podem constituir novos sub-subtipos A. Os dados epidemiológicos disponíveis e a elevada divergência genética intra-subtipo dos isolados Angolanos são consistentes com uma epidemia antiga neste país. Pensamos que a guerra colonial com os Portugueses (1961-1974) terá sido a principal causa da disseminação de VIH-1 em Angola. A epidemia de VIH/SIDA em Portugal (Capítulo 7) e Cabo Verde (Capítulo 5) é causada por uma grande proporção de subtipos não-B e recombinantes, alguns fortemente relacionados com os isolados Angolanos. Deste modo, a grande migração de pessoas durante a guerra colonial com Angola pode também ter promovido a disseminação de subtipos não-B de VIH-1 para Portugal e Cabo Verde, possivelmente em meados dos anos 60, e daí para o resto do mundo. Tendo isto em conta, é provável que Angola tenha sido um dos epicentros da actual epidemia de subtipos não-B em todo o mundo.

Em Maputo, Moçambique, a maior parte (81%) dos doentes estava infectada com vírus de subtipo C de origem diversa (Capítulo 4), contudo, também se detectaram quase todos os outros subtipos. De relevância particular neste contexto, foi a identificação de um *cluster* de isolados CRF37_cpx que terão sido importados recentemente para Moçambique. Em geral, os nossos resultados indicam que a epidemia VIH-1 em Moçambique (Maputo) está a evoluir rapidamente em complexidade genética devido à recente introdução de todos os principais subtipos e formas recombinantes. No futuro será importante determinar de que modo esta alteração da diversidade viral terá impacto na epidemia de VIH/SIDA em Moçambique.

Em Cabo Verde caracterizou-se pela primeira vez as formas genéticas de VIH-1 e de VIH-2 em circulação (Capítulo 5). O subtipo G de VIH-1 foi o subtipo mais prevalente (48%). Encontraram-se ainda outros subtipos bem como variantes não tipáveis, sozinhos ou em formas recombinantes. Os isolados de subtipo G de Cabo Verde são extremamente divergentes em relação aos isolados de referência e na sua maioria parecem ter origem em Portugal e/ou Angola. Isto não é surpreendente visto Cabo Verde ter fortes relações históricas, sociais e económicas com estes dois países. Encontrou-se um recombinante C(*pol*)/G(*env*) ainda não descrito que importa agora sequenciar a nível genómico para determinar se poderá vir a definir uma nova CRF_CG. Todos os isolados VIH-2 pertenciam ao grupo A, um grupo que é também endémico em Portugal e nos países da África Ocidental relacionados com Cabo Verde. Encontraram-se dois isolados VIH-2 que partilhavam um ancestral comum com o histórico VIH-2ROD, o primeiro VIH-2 a ser sequenciado em 1987. Estes

resultados sugerem que a origem do VIH-2 ROD é Cabo Verde e que este país poderá ser ter sido um dos epicentros da actual epidemia de VIH-2.

Não se encontraram mutações de resistência *major* aos inibidores da protease (IPs) na PR dos isolados de VIH-1 presentes em Angola, Moçambique e Cabo Verde (Capítulos 3, 4, 5), o que é consistente com o facto dos regimes de primeira linha utilizados nestes países não inclurem esta classe de fármacos. No entanto, na PR dos isolados não-B foram identificados um grande número de polimorfismos naturais em posições que no subtipo B são consideradas mutações *minor* de resistência aos IPs (exs. L10I/V, V11I e T74P). Adicionalmente, foram encontrados novos polimorfismos ainda não descritos nas bases de dados de sequências de PR referentes a doentes não tratados. Estes resultados confirmam que os vírus que circulam nestes países são extremamente divergentes e sugerem que estes vírus poderão ter uma barreira genética de resistência diminuída para alguns IPs.

Na RT dos isolados VIH-1 de Angola, Moçambique e Cabo Verde também se encontraram numerosos polimorfismos que poderão ter impacto na susceptibilidade aos inibidores da RT (Capítulos 3, 4, 5). Adicionalmente, foram encontradas mutações de resistência aos INRTs (M41L, D67N, M184V, L210W, T215F/Y, K219Q) e INNRTs (K103N) nalguns doentes não tratados de Angola (2 doentes, 2%), Moçambique (4, 6%) e Cabo Verde (3, 12%). Nenhum destes isolados com mutações de resistência foi totalmente sensível aos regimes antirretrovirais de primeira linha usados nestes países. A baixa prevalência de resistência transmitida encontrada em Angola é consistente com a baixa disponibilidade de antirretrovirais no período de estudo (1993-2001). Pelo contrário, as taxas de resistência transmitida encontradas em Moçambique e Cabo Verde foram inesperadas dado que os fármacos antirretrovirais só começaram a estar disponíveis nestes países por volta de 2004. Os nossos resultados sugerem que a maior parte dos isolados resistentes terão sido importados de países onde a terapêutica está disponível há mais tempo. No entanto, o uso não monitorizado de antiretrovirais poderá também ser responsável por estas taxas relativamente elevadas de transmissão de vírus resistentes.

Em Portugal estudámos um caso raro de infecção seronegativa por VIH-1 com rápida progressão para SIDA e morte (Capítulo 6). O doente estava infectado com um vírus recombinante B(*env*)/G(*gag*) semelhante à CRF14_BG encontrada originalmente em Espanha em toxicodependentes. A análise filogenética indicou que o doente tinha sido infectado pela sua parceira sexual, uma toxicodependente, e que havia uma evolução genética muito restrita do vírus. A análise genética do tropismo indicou que o doente foi infectado selectivamente com uma variante CCR5. Estes dados demonstraram que na ausência de pressão selectiva por anticorpos os vírus CCR5 podem provocar SIDA e morte. No global, os resultados sugeriram uma infecção massiva com uma estirpe tipo CRF14_BG extremamente agressiva e/ou a presença de um problema imunológico não identificado no doente que impediu a formação de anticorpos específicos contra VIH-1.

Por sequenciação e análise filogenética do genoma completo de três doentes não relacionados, infectados com recombinantes B/G, fizemos a primeira caracterização molecular e evolutiva dos

vírus CRF14_BG em circulação em Portugal (Capítulo 7). Os nossos resultados sugerem que a CRF14_BG terá emergido em Portugal em 1992 (1989-1996) após o início da epidemia, tendo-se disseminado para Espanha no fim dos anos 90, provavelmente como consequência da migração de toxicodependentes, e depois para o resto da Europa. Verificámos que a grande maioria dos isolados CRF14_BG usa o co-receptor CXCR4 e estão associados à rápida depleção das células CD4 e progressão para SIDA. Finalmente, verificámos que só um aminoácido na V3 *loop* dos CRF14_BG está sobre pressão selectiva enquanto que no subtipo B há quatro aminoácidos sobre pressão selectiva. No conjunto, os resultados sugerem que os vírus CRF14_BG são particularmente patogénicos, que esta característica está em geral associada ao uso do co-receptor CXCR4, e que o uso preferencial deste co-receptor pode resultar de uma evolução convergente determinada pela fuga eficiente à resposta humoral neutralizante.

Abstract

The aims of this thesis were to better characterize HIV-1 diversity in Portugal, Angola, Mozambique and Cape Verde and to investigate the origin and epidemiological history of HIV-1 in these countries. The impact of these issues in diagnosis, disease progression and susceptibility to ARV therapy was also investigated. Finally, the nature, dynamics and prevalence of transmitted drug resistance (TDR) was determined in untreated HIV-1 infected patients.

In Angola, practically all HIV-1 genetic forms were found, including almost all subtypes, untypable (U) strains, CRFs and URFs. Recombinants (first and second generation) were present in 47.1% of the patients. HIV/AIDS epidemic in Angola probably started in 1961, the major cause being the independence war, subsequently spreading to Portugal. In Maputo, 81% of the patients were infected with subtype C viruses. Subtype G, U and recombinants such as CRF37_cpx, were also present. The results suggest that HIV-1 epidemic in Mozambique is evolving rapidly in genetic complexity. In Cape Verde, where HIV-1 and HIV-2 co-circulate, subtype G is the prevailed subtype. Subtypes B, C, F1, U, CRF02_AG and other recombinant strains were also found. HIV-2 isolates belonged to group A, some being closely related to the original ROD isolate. In all three countries numerous new polymorphisms were identified in the RT and PR of HIV-1 viruses. Mutations conferring resistance to the NRTIs or NNRTIs were found in isolates from 2 (2%) patients from Angola, 4 (6%) from Mozambique and 3 (12%) from Cape Verde. None of the isolates containing TDR mutations would be fully sensitive to the standard first-line therapeutic regimens used in these

countries. Close surveillance in treated and untreated populations will be crucial to prevent further transmission of drug resistant strains and maximize the efficacy of ARV therapy.

In Portugal, investigation of a seronegative case infection with rapid progression to AIDS and death revealed that the patient was infected with a CRF14_BG-like R5-tropic strain selectively transmitted by his seropositive sexual partner. The results suggest a massive infection with a highly aggressive CRF14_BG like strain and/or the presence of an unidentified immunological problem that prevented the formation of HIV-1-specific antibodies. Near full-length genomic sequences obtained from three unrelated patients enabled the first molecular and phylogenomic characterization of CRF14_BG from Portugal; all sequences were strongly related with CRF14_BG Spanish isolates. The mean date of origin of CRF14_BG was estimated to be 1992. We propose that CRF14_BG emerged in Portugal in the early 1990s, spread to Spain in late 1990s as a consequence of IDUs migration and then to the rest of Europe. Most CRF14_BG strains were predicted to use CXCR4 and were associated with rapid CD4 depletion and disease progression. Finally, we provide evidence suggesting that the X4 tropism of CRF14_BG may have resulted from convergent evolution of the V3 loop possibly driven by an effective escape from neutralizing antibody response.

Abbreviations

3TC	lamivudine
ABC	abacavir
AIDS	acquired immunodeficiency syndrome
APV	amprenavir
ART	antiretroviral
ATV	atazanavir
AZT	zidovudine
bDNA	branched-chain DNA signal amplification
CA	coreceptor antagonist
cART	combined antiretroviral therapy
CCR5	C-C chemokine receptor type 5
CD4bs	CD4 binding site
cpx	complex
CRF	circulant recombinant form
CXCR4	C-X-C chemokine receptor type 4
DBS	dried blood spots
ddI	didanosine
d4T	stavudine
DDC	zalcitabine
DRC	Democratic Republic of Congo
DRM	drug resistance mutation

DRV	darunavir
dS	synonymous substitution
EFV	efavirenz
EI	entry inhibitor
ETR	etravirine
fAPV	fosamprenavir
FDA	Food and Drug Administration
FI	fusion inhibitor
FTC	emtricitabine
HAART	highly active antiretroviral therapy
HIV-1	human immunodeficiency virus type 1
HIV-2	human immunodeficiency virus type 2
HLA	human leukocyte antigen
HR1	heptad repeat 1
HR2	heptad repeat 2
HTLV	human T cell lymphotrophic virus
IDU	intravenous drug user
IDR	immunodominant region
IDV	indinavir
II	integrase inhibitor
INT	integrase
LPV	lopinavir
M	major or main HIV-1 group
mAb	monoclonal antibody
MVC	maraviroc
MSM	men who have sex with men
MTCT	mother-to-child transmission
N	non-M non-O HIV-1 group
NAb	neutralizing antibody
NAAT	nucleic acid amplification testing
NASBA	nucleic acid sequence-based amplification
NFV	nelfinavir
NNI	nearest-neighbor interchange
NNRTI	non-nucleoside reverse transcriptase inhibitor
NRTI	nucleos(t)ide reverse transcriptase inhibitor
NVP	nevirapine
O	outlier HIV-1 group
P	P HIV-1 group

PCR	polymerase chain reaction
PENTA	Pediatric European Network for Treatment of AIDS
PI	protease inhibitor
PR	protease
RAL	raltegravir
qRT-PCR	quantitative real time polymerase chain reaction
RT	reverse transcriptase
RT-PCR	reverse transcriptase polymerase chain reaction
(RT) PCR	real time polymerase chain reaction
RTV	ritonavir
sdNVP	single dose nevirapine
SQV	saquinavir
SIV	simian immunodeficiency virus
T20	enfurvitide
TARc	tenofovir
TDF	tenofovir
TDR	transmitted drug resistance
TPV	tipranavir
U	untypable or unclassified
URF	unique recombinant form
USA	United States of America
WHO	World Health Organization

Table of Contents

CHAPTER 1

General Introduction

Acquired Immunodeficiency Syndrome

Acquired immunodeficiency syndrome (AIDS) is a complex disease of the human immune system caused by the human immunodeficiency virus type 1 (HIV-1) and type 2 (HIV-2) [1]. AIDS was first recognized as a disease in the United States in 1981 after the observation of a high incidence of rare opportunistic infections in homosexual men caused by a general immune deficiency [2]. HIV-1 was isolated from this population in 1983 [3, 4]; HIV-2, which has a slightly different genomic structure [5], was isolated from West African AIDS patients in 1985 [6]. HIV-1 is responsible for the global pandemic while HIV-2, that appears to be less pathogenic [9, 10], is mainly restricted to West Africa [7] and countries with historical links to that area, like Portugal [8].

The overall growth of the global AIDS epidemic appears to have stabilized [11]. The annual number of new HIV infections has been steadily declining since the late 1990s and there are fewer AIDS-related deaths due to the significant scale up of antiretroviral (ARV) therapy over the past few years [11]. By the end of 2009 an estimated 33.3 million adults and children were living with HIV/AIDS worldwide. More than 67% (22.5 millions) of these individuals resided in Sub-Saharan Africa where the average prevalence of HIV infection among adults was 5.0%. In 2009, there were an estimated 2.6 million people who became newly infected with HIV and 1.8 million AIDS-related deaths among adults and children [11].

The introduction of highly active antiretroviral therapy (HAART), in developed countries in 1996 and in developing countries in 2004, has caused a significant decrease in the mortality and morbidity rates associated with HIV-1 infection and, the prognosis for patients who have access to these drugs has also improved significantly [12-16].

Genetic Diversity of HIV-1

HIV-1 is characterized by a very high rate of molecular evolution which allows the virus to diversify into numerous genetic forms, escape the human immune system and develop drug resistant variants [17]. HIV-1 can diversify by at least two ways: by mutation (substitutions, deletions, and insertions) occurring during viral DNA synthesis by the viral reverse transcriptase (RT) due to its lack of DNA proofreading activity; and by recombination, which is due to the RT strand-transfer activity and occurs in cells infected simultaneously with two or more virus strains [18-22].

The mutation rate of HIV-1 is 2.4×10^{-5} nucleotide substitution per nucleotide per cell infection [21] and recombination occurs at an estimated rate of at least 2.8 crossovers per genome per cycle [22]. The effective recombination rate, i.e., the product of super-infection and crossovers, is 1.4×10^{-5} recombinations per site and generation [23]. In nature, the high genetic diversity of HIV is enhanced by a high level of virus production (10^8 to 10^{10} virions per day) [24-26], a large number of viral replication cycles (10^7 to 10^8 cycles per day) [24, 25], and a large pool of infected individuals. Within its host HIV exists as a pool of closely related genetic variants, known collectively as quasispecies [27].

Origin and Classification of HIV

HIV-1 and HIV-2 are distinguished on the basis of their genome organizations and phylogenetic (i.e., evolutionary) relationships with other primate lentiviruses. The two human viruses are related to different simian immunodeficiency viruses (SIVs) and therefore have different evolutionary origins [17]. HIV-1 is more related to SIV isolated from chimpanzees (SIVcpz) [28-31] and from gorillas (SIVgor) [32] and HIV-2 is more closely related to SIVsm, which is found with high prevalence in sooty mangabey monkeys [33].

HIV entered the human population as a result of zoonotic, or cross-species, transmission [31, 34, 35]. Phylogenetic analysis shows that there have been many cross-species transmissions to humans; for instance in HIV-2 this number might be at least eight [34], whereas the independent origin of HIV-1 groups M (major or main), N (non-M, non-O) and O (outlier) could be explained by three jumps from chimpanzees to humans [36, 37]. Recently a new human immunodeficiency virus closely related to SIVs from gorillas, SIVgor, was identified [32]. This new HIV-1 variant is distinct from the three established groups of HIV-1, namely M, N, and O and was designated HIV-1 group P (Fig. 1.1).

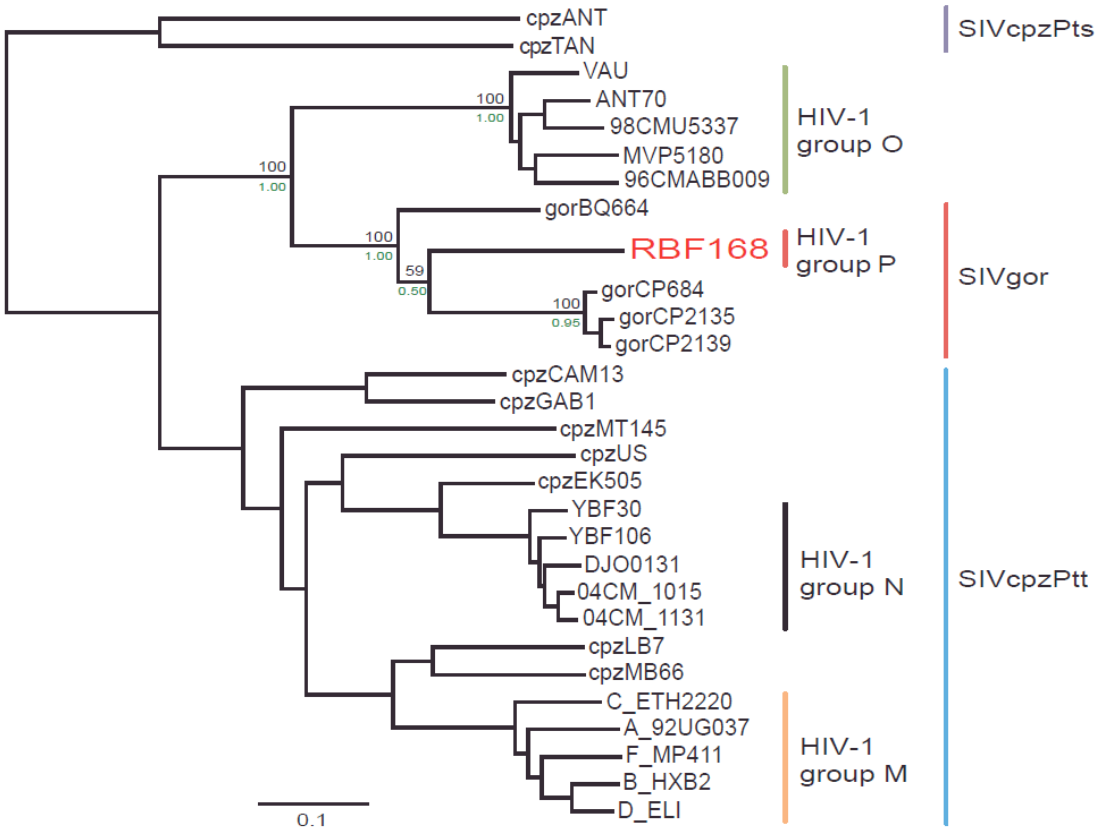

Figure 1.1 - Evolutionary relationship between the HIV-1 different groups. The unique isolate of HIV-1 group P is shown in red letters. Adapted from Plantier *et al.*, Nat Med 2009 [32].

HIV-1 groups N and P seem to be restricted to Cameroon and have few documented cases [32, 37-39]. Group O viruses have been identified in persons with epidemiological links to Central Africa, mainly Cameroon and some neighboring countries [40].

The earliest documented cases of HIV-1 group M infection, dating from 1959 and 1960, were identified in patients from the Democratic Republic of Congo (DRC) (former Zaire) [41, 42]. Phylogenetic analyses put the 1959 sequence closest to the ancestral node of subtype D and the 1960 sequence closest to subtype A [42]. These data showed that diversification of HIV-1 in West-Central Africa took place long before the recognition of the AIDS pandemic. Using different methods of molecular clock analysis it was estimated that group M was originated in the 1930s (1915-1941) [42-45]. For HIV-2, the date of the most recent common ancestor of group A strains was estimated to be 1940 (1924-1956), and that of B strains was estimated to be 1945 (1931-1959) [46].

Since its emergence, HIV-1 group M has diversified into nine subtypes, A, B, C, D, F, G, H, J, and K, seven sub-subtypes (A1-A5 and F1-F2), multiple circulating recombinant forms (CRFs) and countless unique recombinant forms (UFRs) [47-51]. Subtypes are genetically equidistant from one another, with the intersubtype nucleotide distances ranging from 11 to 17% in the *gag* gene and from 18 to 22% in the *env* gene. Intrasubtype distances range from 5 to 7% in the *gag* gene and 11 to 14% in the *env* gene. Intersub-subtype diversity ranges from 10 to 12% in the *gag* gene and from 16 to 17% in the *env* gene. CRFs are recombinant forms that have become epidemic [52-54]. Like subtypes, CRFs are defined by significant clustering in phylogenetic analysis and, additionally, by an identical recombinant structure. At present, 49 CRFs were recognized of which 37 are first generation recombinants (Table 1.1) and 12 are second generation recombinants (Table 1.2) [54]. Second generation CRFs result from recombination between more than one first generation CRF or from recombination between CRFs and different subtypes (Table 1.2) [53, 55-67]. CRFs are described by their specific structure and by the subtypes they contain; when more than three subtypes are present, the designation ‘‘cpx’’ (complex) is used [53]. URFs are recombinant forms that were identified in a single individual and therefore have a unique subtype composition and recombinant structure. The URFs represent a large and heterogeneous group; they usually reflect the mixture of subtypes in the population where they are found, and some may be generated within individuals with a dual HIV-1 infection [68, 69].

For HIV-2 there are eight recognized groups (A-H). HIV-2 groups A and B have infected a substantial number of people in West Africa, while groups C to H have each been identified only in single individuals [70-73].

TABLE 1.1

First Generation HIV-1 Circulant Recombinant Forms

Name	Subtypes	Reference
CRF01_AE	A, E	[74]
CRF02_AG	A, G	[75]
CRF03_AB	A, B	[76]
CRF04_cpx	A, G, H, K, U	[77]
CRF05_DF	D, F	[78]
CRF06_cpx	A, G, J, K	[79]
CRF07_BC	B', C	[80]
CRF08_BC	B', C	[81]
CRF09_cpx	A, G, U	[81, 82]
CRF10_CD	C, D	[83]
CRF12_BF	B, F	[84]
CRF14_BG	B, G	[85]
CRF16_A2D	A2, D	[86]
CRF17_BF	B, F	[84]
CRF18_cpx	A1, F, G, H, K, U	[87]
CRF19_cpx	A1, D, G	[88]
CRF20_BG	B, G	[89]
CRF21_A2D	A2, D	[90]
CRF23_BG	B, G	[89]
CRF24_BG	B, G	[89]
CRF25_cpx	A, G, U	[91]
CRF26_AU	A, U	[51]
CRF27_cpx	A, E, G, H, J, K, U	[92]
CRF28_BF	B, F	[93, 94]
CRF29_BF	B, F	[93, 94]
CRF31_BC	B, C	[95]
CRF35_AD	A, D	[96]
CRF38_BF	B, F1	[97]
CRF39_BF	B, F1	[98]
CRF40_BF	B, F	[98]
CRF41_CD	C, D	*

TABLE 1.1 *(continued)*

Name	Subtypes	Reference
CRF44_BF	B, F1	[99]
CRF45_cpx	A, K, U	[100]
CRF46_BF	B, F1	[101]
CRF47_BF	B, F1	[102]
CRF49_cpx	A1, C, J, K, U	[103]

* Not yet published.

TABLE 1.2

Second Generation HIV-1 Circulant Recombinant Forms

Name	Subtypes	Reference
CRF11_cpx	A, CRF01, G, J	[59]
CRF13_cpx	A, CRF01, G, J, U	[65]
CRF15_01B	CRF01, B	[55]
CRF22_01A1	CRF01, A1	[104]
CRF30_0206	CRF02, CRF06	[58]
CRF32_06A1	CRF06, A1	[56]
CRF33_01B	CRF01, B	[62]
CRF34_01B	CRF01, B	[63]
CRF36_cpx	A, G, CRF01, CRF02	[61]
CRF37_cpx	A, G, CRF01, CRF02, U	[60]
CRF43_02G	CRF02, G	[66]
CRF48_01B	CRF01, B	[57]

Geographic Distribution

In Table 1.3 the main geographic distribution of HIV-1 subtypes and recombinants are listed, along with an estimate of relative global prevalences. Five HIV-1 strains dominate the global epidemic: subtypes A, B, and C, along with CRF01_AE and CRF02_AG, with subtype C accounting for almost 50% of all HIV-1 infections worldwide (Table 1.3) [52, 105-107]. Molecular epidemiological studies show that, with the exception of sub-Saharan Africa where almost all subtypes, CRFs, and several URFs have been detected, there is a specific geographic distribution pattern for HIV-1 subtypes

(Table 1.3) [52, 105-107]. Subtype A is prevalent in Central and Eastern Africa (Kenya, Uganda, Tanzania, and Rwanda) [108-110], Iran [111], Eastern Europe [112, 113], and Central Asia [114, 115]. In all these cases, sub-subtype A1 is more prevalent, whereas sub-subtypes A2-A5 are primarily found in Africa and rarely in Europe.

Subtype B is the most disseminated variant. It predominates in developed countries, such as United States of America (USA) and Canada [116-119], in Brazil [95, 120, 121], countries of Western and Central Europe [122-130] and Australia [131, 132], and is also common in several countries of Southeast Asia [133, 134], northern Africa [135] and the Middle East [136], and among South African and Russian homosexual men [52, 105-107, 137]. The global spread of subtype B is considered a major event in the history of HIV/AIDS because it marks the point when the virus first entered the large, wealthy and highly mobile populations of the Western world. Gilbert and collaborators estimated that the HIV-1 subtype B entered in Haiti from Africa around 1966 (1962-1970) [138]. This timescale corresponds well with a period when many Haitians returned to their home country from the DRC, after the latter's independence from Belgium and subsequent political crises. The migration of HIV-1 subtype B from Haiti to the United States and beyond is dated to the 1966–1972 period (1969) [138], suggesting that HIV-1 was circulating cryptically in the USA for approximately 12 years before the initial recognition of AIDS in 1981 [2]. The introduction of HIV-1 subtype B into Europe occurred mainly through homosexual contacts or needle sharing in or from the USA [139-141], or through heterosexual contacts with individuals from Central Africa [142-144].

Subtype C is the overwhelming prevailing strain in Southern Africa [145-148], in India and neighbor countries [149, 150] and in the southern region of Brazil [121]. Subtype D strains are found mainly in East Africa, and to a lesser extent in West Africa [78, 108, 110, 151]. Subtype F predominates in Central Africa [152, 153], South America [154, 155] and Eastern Europe [156, 157]. Subtype G viruses are prevalent in Central and Western Africa [158, 159], as well as in Portugal [122, 160-162] and Spain [124, 163]. Subtypes H and J were described in Central Africa [164-166] and in Angola [167, 168]. Subtype K was identified in DRC and Cameroon [169].

Some CRFs have high impact in local AIDS epidemics, such as CRF01_AE in Southeast Asia [170, 171] and CRF02_AG in Western and Central Africa [172-174]. CRF01_AE represents a putative subtype A/E recombinant that is spreading epidemically in Asia, but originated from Central Africa [74, 170]. No "pure" full-length genome has been found for subtype E. CRF02_AG [175] is a subtype A/G recombinant form that is highly prevalent in West and Central Africa [75], but has also been reported in Taiwan [176]. CRF03_AB represents a subtype A/B recombinant that was first found in Kaliningrad, and is circulating in Russian and Ukrainian cities, primarily in injecting drug users (IDUs) [76, 177]. Other CRFs linked to this transmission group (IDUs) are: CRF07_BC and CRF08_BC in China [80, 81, 178-180], CRF14_BG in Portugal and Spain [85, 161, 181], CRF32_06A1 in Estonia [56], CRF33_01B [62] and CRF4801_B in Malaysia [57], CRF15_01B [55] and CRF34_01B in Thailand [63] and CRF35_AD in Afghanistan [96].

The first B/G recombinant form – CRF14_BG - was described in Spanish and Portuguese IDUs [85, 161, 181]. This CRF was first isolated in 2002 from IDUs in Galiza, Spain [85]. So far, only 10 CRF14_BG isolates have been characterized by full-genome sequencing. These were obtained from Spanish (5/10, 50%), and German (1, 10%) IDUs, and from Portuguese patients, one IDU, one patient infected by heterosexual transmission and two children infected by perinatal transmission [85, 182, 183].

Until 2007, several sub-genomic sequences related to CRF14_BG were reported in Germany (1), Italy (2), United Kingdom (2), Estonia (15), Spain (38) and Portugal (50) suggesting that this CRF spread efficiently throughout Europe [56, 161, 162, 181, 182, 184-195]. The other B/G recombinant forms identified so far, CFR20_BG, CRF23_BG and CRF24_BG, were described in Cuban patients, [89, 196]. Two complex recombinants with an African origin, CRF18_cpx [87] and CRF19_cpx [88], were also first described in Cuba. The pronounced genetic complexity of HIV-1 in Cuba is not unexpected, considering that large numbers of Cuban military and civilian personnel had been stationed in the 1960s and 1970s in several African countries [197], and when they returned home probably brought with them different genetic forms of HIV-1 present in those countries [167, 168, 198].

In South America (Argentina, Cuba, Bolivia, Brazil, Chile, and Uruguay) the epidemic is a mixture of subtype B and B/F recombinants [84, 93, 97-99, 101, 199, 200] with a small proportion of infections by subtype C [121]. CRF42_BF described in Luxembourg and CRF47_BF described in Spanish patients, were the first B/F1 CRFs to be identified outside South America [84, 93, 97-99, 101, 102, 200, 201]. The 21 clinical isolates of CRF42_BF form a cluster clearly distinct from previously described South American CRF_BF strains [201]. The subtype F segments from CRF47_BF are distinct from South American subtype F1 sequences [102].

CRF11_cpx was the first second generation CRF described in 2000 in patients from Cameroon [202]. This CRF circulates in Cameroon, Central African Republic, Gabon, DRC, and Angola although its exact prevalence rate remains to be determined [67, 168, 203-207]. Second generation CRFs are becoming common in complex epidemics with multiple subtypes and recombinant forms. At present they have been detected in Africa, East Asia, Thailand, Malaysia, Estonia and Saudi Arabia (Table 1.3).

HIV-1 group O seems to be endemic in Cameroon and neighboring countries in West-Central Africa and represents only about 1-5% of HIV-1 positive samples in this region [208, 209]. Elsewhere in the world, group O viruses have been identified mainly from people with epidemiological links to the referred Central African countries [40]. HIV-1 groups N and P circulate exclusively in Cameroon [32, 38, 210, 211]. In some regions of the world, little information is available about HIV diversity, particularly in North Africa, the Middle East, and parts of Central Asia.

TABLE 1.3

HIV-1 Group M Subtypes and CRFs in the Global Epidemic

HIV-1 Genetic Forms		Main Geographic Localizations	Global Prevalence (%)	Reference
Subtypes	A	Central and Eastern Africa (Kenya, Uganda, Tanzania, and Rwanda), Iran, Eastern Europe and Central Asia	12.3	[108-115]
	B	America, Western and Central Europe, Australia, and is also common in several countries of Southeast Asia, Northern Africa and Middle East	10.4	[52, 95, 105-107, 116-136, 212]
	C	Southern Africa, India and neighbor countries and Southern region of Brazil	49.9	[121, 145-150]
	D	East and West Africa	2.5	[78, 108, 110, 151]
	F	Central Africa, South America and Eastern Europe	0.6	[152-157]
	G	Central and Western Africa, Portugal and Spain	6.3	[122, 124, 158-163]
	H	Central Africa and Angola	0.17	[164, 165, 167, 168]
	J	Central Africa and Angola	0.14	[164-168]
	K	DRC and Cameroon	0.04	[169]
1st Generation	CRF01_AE	Southeast Asia	4.7	[74, 170, 171]
CRFs	CRF02_AG	West and Central Africa and Taiwan	6.7	[75, 172-174, 176]
	CRF03_AB	Former Soviet Republics	0.1	[76, 177]
	CRF04_cpx	Cyprus and Greece	0.003	[77, 213, 214]
	CRF05_DF	Central Africa	0.0001	[78]
	CRF06_cpx	West Africa	0.005	[215, 216]
	CRF07_BC	China	0.005	[80, 178, 179]
	CRF08_BC	China	0.001	[81, 178, 179]

TABLE 1.3 *(continued)*

HIV-1 Genetic Forms		Main Geographic Localizations	Global Prevalence (%)	Reference
1st Generation CRFs	CRF09_cpx	West Africa and Angola	0.0003	[81, 82]
	CRF10_CD	East Africa (Tanzania, Madagascar)	0.0007	[83, 217, 218]
	CRF12_BF	Argentina, and Uruguay	0.001	[84, 97]
	CRF14_BG	Portugal and Spain	0.0006	[122, 161, 181]
	CRF16_A2D	Kenya, South Korea, and Argentina	0.00005	[86, 219]
	CRF17_BF	Argentina, Uruguay, and Bolivia	0.00002	[84]
	CRF18_ cpx	Cuba	0.0003	[87, 220]
	CRF19_ cpx	Cuba	0.0003	[88, 220]
	CRF20_BG	Cuba	0.00007	[89, 196]
	CRF21_A2D	Kenya	<0.00001	[86, 90]
	CRF23_BG	Cuba	0.00005	[89, 196]
	CRF24_BG	Cuba	0.00009	[89, 196]
	CRF25_cpx	Cameroon, DRC, and Saudi Arabia	0.00003	[66, 91, 207]
	CRF26_AU	DRC	0.00004	[51, 207]
	CRF27_cpx	DRC	0.00003	[92]
	CRF28_BF	Brazil	<0.00001	[93, 94]
	CRF29_BF	Brazil	0.00001	[93, 94]
	CRF31_BC	Brazil	0.00006	[95]
	CRF35_AD	Afghanistan, and Iran	0.0001	[96, 221]
	CRF38_BF	Uruguay	<0.00001	[97]
	CRF39_BF	Brazil	<0.00001	[98]

TABLE 1.3 *(continued)*

HIV-1 Genetic Forms		Main Geographic Localizations	Global Prevalence (%)	Reference
1st Generation CRFs	CRF40_BF	Brazil	0.00002	[98]
	CRF41_CD	*	*	*
	CRF42_BF	Luxembourg	0.00006	[200, 201]
	CRF44_BF	Chile	0.00004	[99]
	CRF45_cpx	Cameroon, Gabon, and DRC	0.00006	[100]
	CRF46_BF	Brazil	0.00003	[101]
	CRF47_BF	Spain	0.00003	[102]
	CRF49_cpx	Gambia	<0.00001	[103]
2nd Generation CRFs	CRF11_cpx	Central Africa (Cameroon, Central African Republic, Gabon, and DRC)	0.003	[59, 203-207]
	CRF13_cpx	Central and Southern Africa (Cameroon, Angola and DRC)	0.0007	[65, 168, 207, 222]
	CRF15_01B	Thailand	0.00006	[55, 64]
	CRF22_01A1	Cameroon	0.0001	[67]
	CRF30_0206	Nigeria	<0.00001	[58]
	CRF32_06A1	Estonia	<0.00001	[56]
	CRF33_01B	Malaysia	0.0001	[62, 134]
	CRF34_01B	Thailand	0.00002	[63]
	CRF36_cpx	Cameroon	0.00005	[61]
	CRF37_cpx	Cameroon, Mozambique and DRC	0.00008	[60, 148, 207]
	CRF43_02G	Saudi Arabia	0.00003	[66]
	CRF48_01B	Malaysia	<0.00001	[57]

[1]The global prevalence of each form is expressed as a percentage of the total number of HIV-1 isolates identified worldwide. Isolates from HIV-1 groups O and N were not included in this table. * Not yet been published.

Antiretroviral Therapy and Resistance

Since the advent of HAART in developed countries in 1996, and in developing countries in 2004 we have seen an improved prognosis of HIV-1 infected patients, with a significant decrease in morbidity and mortality rates associated with this infection [12-16]. The currently available treatment provides better quality of life to HIV/AIDS patients, minimizing viral replication, promoting partial recovery of the immune system and, thus, delaying or halting disease progression [223].

Antiretroviral Drugs

In March of 1987, zidovudine (AZT) was the first HIV-1 drug approved by the USA Food and Drug Administration (FDA) for AIDS treatment. Between 1991 and 2008, 25 additional drugs were approved [224]. These drugs fall into five categories, according to the viral protein target (Table 1.4). Currently, the standard treatment is a combination of drugs, named HAART. It consists of two nucleos(t)ide reverse transcriptase inhibitors (NRTIs) and a non-nucleoside reverse transcriptase inhibitor (NNRTI) or a protease inhibitor (PI) [225]. Due to high costs, more recent ARV drug classes, such as entry inhibitors (EIs) and integrase inhibitors (IIs), are primarily used for salvage therapy in cases of multiple resistances to the previous classes [225].

Nucleos(t)ide Reverse Transcriptase Inhibitors (NRTIs)

The target of NRTIs is the RT. There are eight NRTIs (Table 1.4). NRTIs are phosphorylated by cellular enzymes and converted into their active NRTI triphosphate form. Once activated, they compete with the natural nucleoside triphosphates for binding the RT polymerase active site, and after their incorporation into the primer strand, act as terminator of DNA synthesis due to the lack of a 3'-hydroxyl group [226]. NtRTIs (tenofovir) should be clearly distinguished from the NRTIs as they are nucleotide analogues (not nucleoside analogues), which means that they only need two (not three) phosphorylation steps to be converted into their active form [224].

Non Nucleoside Reverse Transcriptase Inhibitors (NNRTIs)

There are at present four NNRTIs licensed for clinical use in the treatment of HIV infections (Table 1.4). NNRTIs are designed to bind to an RT hydrophobic pocket (binding site), located in the palm domain of the p66 subunit at a close distance from the active (catalytic) site of HIV-1 RT, modifying its structure allosterically and impairing the polymerase domain catalytic site, thereby exerting a non-competitive inhibition [227].

New strategies to inhibit RT enzymatic activities and to overcome viral resistance are under investigation: a new NNRTI, rilpivirine (TMC278) is currently in Phase III clinical trials [228].

TABLE 1.4

Antiretroviral Drugs in Clinical Use

Drug Class	Drug	Release Year
Nucleos(t)ide reverse transcriptase inhibitors (NRTIs)	Zidovudine (AZT)	1987
	Didanosine (ddI)	1991
	Zalcitabine (ddC)	1992
	Stavudine (d4T)	1994
	Lamivudine (3TC)	1995
	Abacavir (ABC)	1998
	Tenofovir (TDF)	2001
	Emtricitabine (FTC)	2003
Non-nucleoside reverse transcriptase inhibitors (NNRTIs)	Nevirapine (NVP)	1996
	Delavirdine (DLV)	1997
	Efavirenz (EFV)	1998
	Etravirine (ETR)	2008
Protease inhibitors (PIs)*	Saquinavir (SQV)	1995
	Indinavir (IDV)	1996
	Nelfinavir (NFV)	1997
	Amprenavir (APV)	1999
	Lopinavir (LPV)	2000
	Atazanavir (ATV)	2003
	Fosamprenavir (fAPV)	2003
	Tipranavir (TPV)	2005
	Darunavir (DRV)	2006
Entry inhibitors (EIs)		
Fusion inhibitor (FI)	Enfuvirtide (T-20)	2003
Coreceptor antagonist (CA)	Maraviroc (MVC)	2007
Integrase inhibitor (II)	Raltegravir (RAL)	2007

* All PIs are boosted with Ritonavir (RTV) with exception of ATV.

Protease Inhibitors (PIs)

There are ten PIs presently available for the treatment of HIV infections (Table 1.4). PIs are mimetics of viral peptides and bind to the active site of the protease (PR) enzyme, preventing viral maturation in a late step of the virus life cycle, [229].

Entry Inhibitors (EIs)

There are three crucial steps for entry of HIV into the CD4+ T cells: binding of HIV to the CD4 receptor, binding to coreceptors (CCR5 and/or CXCR4), and fusion of virus and cell. There are only two EIs currently available for the treatment of HIV infection, enfuvirtide (T20) – a fusion inhibitor, and maraviroc (MVC) – a CCR5 antagonist [230, 231] (Table 1.4). T20 is a polypeptide of 36 amino acids that is homologous to the heptad repeat 2 (HR2) region of the viral glycoprotein gp41. T20 interacts with HR1 region of gp41 and as a consequence of this interaction, fusion of the virus particle with the outer cell membrane is blocked [232]. MVC interacts with the coreceptor CCR5 used by R5-tropic HIV strains to enter the target cells. MVC inhibits the binding of gp120 to CCR5 by changing its shape such that gp120 can no longer recognize it [233].

Integrase Inhibitor (II)

Raltegravir (RAL) is the only integrase inhibitor available (Table 1.4); it binds to the viral integrase (INT) and prevents the integration of the viral double-stranded cDNA into the host cellular genome during the early steps of the virus cycle [234].

Antiretroviral Regimens

According to UNAIDS, in the end of 2009, there were 15 million of people that needed ARV therapy in low- and middle-income countries [11]: only 36% of those were receiving it. The ARV regimens recommended in these countries are the following [235]: first line regimens - one NNRTI + two NRTIs, one of which should be AZT or TDF; second line regimens - one PI RTV-boosted (ATV/RTV or LPV/RTV) + two NRTIs, one of which should be AZT or TDF.

By the end of 2008, only 2% of the adults in ARV treatment, in low- and middle-income countries, were receiving second-line regimens [236].

In developed countries the recommended ARV regimens should be [225]: first line regimens - two NRTIs (TDF+FTC or ABC+3TC) + one NNRTI (EFV); two NRTIs (TDF+FTC or ABC+3TC) + one PI (ATV/RTV); second line regimens - two NRTIs (TDF+FTC or ABC+3TC) + one PI (DRV/RTV). As an alternative, the third drug in an ARV regimen could be, LPV/RTV, fAPV/RTV or MVC.

Resistance to Antiretroviral Drugs

Management of HIV infection has greatly improved because of the development of new treatment protocols, involving the combination of highly potent drugs [12-16]. However, combination therapy is not effective in all patients and drug resistance is the inevitable consequence of incomplete suppression of HIV replication. Severe side effects, nonadherence to ARV regimens, pharmacokinetic interactions and lack of potency of drugs, are some factors that may contribute to the emergence of resistant viruses [237-240].

Generally, there are two main processes leading to resistance related to treatment failure: resistant viruses may preexist at low frequencies in drug naive patients and are rapidly selected in the presence of ARV drugs (primary or transmitted drug resistance), or, resistant viruses are absent at the start of therapy but are generated by residual viral replication during therapy (secondary or acquired drug resistance) [241].

Currently, drug resistance mutations (DRM) have been described for all drugs in clinical use (Appendix Figure 1) [242]. In the polymerase domain of RT, 15 positions were correlated with loss of drug susceptibility to NRTI, and 14 positions to NNRTI (Appendix Figure 1). In the PR region, 35 positions interfere with PI susceptibility (Appendix Figure 1). Seven positions in gp41 are associated with FIs resistance and four positions in the viral integrase are related to integrase inhibitor resistance (Appendix Figure 1) [242].

There are two types of DRMs, major or primary mutations and minor or secondary mutations, also called compensatory or accessory. Major mutations normally are close to the catalytic site of an enzyme and a single mutation can lead to loss of susceptibility to one or more ARV drugs. Sometimes, one mutation alone can lead to cross resistance to all ARVs within a given drug class [242]. The mutated enzyme is less fit than the wild-type [243, 244]. Minor DRMs could help the mutated enzyme to recover fitness (increase replicative capacity) [245, 246]. However, two or more minor DRMs can lead to loss of drug susceptibility [242]. Some mutations considered major to one ARV can be considered minor DRMs to other drugs [242].

Transmitted Drug Resistance

Transmitted drug resistance (TDR) is a major public health problem, especially in resource limited settings as it can determine rapid loss of effectivity of first line ARV regimens at the population level. Drug naive individuals that acquired a virus with DRMs begin ARV therapy with a lower genetic barrier to resistance, a higher risk of virologic failure and a higher risk of developing resistance even to those drugs in their regimen that were originally fully active [247, 248]. The recent initiation of ARV treatment programs in resourced limited countries [236] highlights the importance of studying this problem. A list of DRMs for surveillance of TDR is available since 2009 (Table 1.5) [249].

In Europe and North America, surveillance studies have reported prevalences of TDR ranging from 8% to 15% in newly diagnosed individuals [250, 251]. In Europe, contrary to USA, TDR prevalence seems to be stabilizing [250-253]. In most Sub-Saharan [254-259] and South and South-East Asia countries [260, 261] TDR is still residual (<5%). This low prevalence is consistent with the very recent introduction of ARVs in these regions [236].

Recently three pathways were proposed to describe the evolution of resistant viruses after transmission to a new host [262]. In the first pathway, the mutation reverts to wild-type when the replicative capacity of the virus is severely diminished; in the second pathway there is a replacement of mutations by atypical amino acids that also improve viral replication capacity; in the third pathway the mutations are fixated because they do not alter significantly viral replication

[262]. This indicates that viruses with these mutations can persist in patients and then be transmitted to new individuals. It is therefore important to continue monitoring the appearance of resistance mutations in treated patients and the rate of transmission in newly diagnosed untreated patients.

TABLE 1.5

Mutations Associated with Transmitted HIV-1 Drug Resistance

NRTIS		NNRTIs		PIs	
Wild type	**Mutation**	**Wild type**	**Mutation**	**Wild type**	**Mutation**
M41	L	L100	I	L23	I
K65	R	K101	E/P	L24	I
D67	N/G/E	K103	N/S	D30	N
T69	D/ins	V106	M/A	V32	I
K70	R/E	V179	F	M46	I/L
L74	V/I	Y181	C/I/V	I47	V/A
V75	M/T/A/S	Y188	L/H/C	G48	V/M
F77	L	G190	A/S/E	I50	V/L
Y115	F	P225	H	F53	L/Y
F116	Y	M230	L	I54	V/L/M/A/T/S
Q151	M			G73	S/T/C/A
M184	V/I			L76	V
L210	W			V82	A/T/F/S/C/M/L
T215	Y/F/I/S/C/D/V/E			N83	D
K219	Q/E/N/R			I84	V/A/C
				I85	V
				N88	D/S
				L90	M

Impact of HIV-1 Genetic Diversity

Transmission

Differential characteristics of viral subtypes and their interactions with the human host may influence HIV transmission and disease progression.

A recent study performed in HIV-discordant couples in Uganda found that subtype A had a significant higher rate of heterosexual transmission than subtype D (P=0.01) and recombinant viruses also had a higher rate of transmission than subtype D, however without statistical significance. This could indicate that subtype D may be less transmissible during heterosexual

contact than non-D viruses [263]. The rate of transmission may reflect differences in subtype-specific coreceptor tropism. HIV-1 can use as coreceptors CCR5 (R5 variants), CXCR4 (X4 variants) or both (R5X4 variants or dual tropic) to enter the cells [264]. R5 variants are largely prevalent during primary infection. Moreover, even when both R5 and X4 variants are present in the donor, most often only the R5 variants are detected in the recipient. Together, these data indicate that R5 strains are more easily transmitted or established in the newly infected host than X4 strains [264]. An increased prevalence of X4 variants has been reported for subtype D [265-267]. Thus, the higher frequency of X4 tropism in subtype D viruses may in part explain their reduced transmissibility when compared to other genetic forms [263].

Although earlier studies found an association between CRF01_AE and heterosexual transmission and between subtype B and intravenous drug use [170, 268], a more recent longitudinal study performed in Thailand found an increased probability of CRF01_AE transmission among IDUs compared with subtype B [269]. Subtype A is linked to heterosexual contact in Africa [108-110] but in European Eastern countries circulates among IDUs [112, 113].

Several studies have looked for variations between clades in mother-to-child HIV-1 transmission (MTCT) rates. In Kenya, MTCT appeared to be more common among mothers infected with subtype D compared with subtype A (P=0.002) and this association was independent of other risk factors for MTCT, such as maternal HIV viral load, episiotomy or perineal tear, and low birth weight [270]. On the other hand in Tanzania, HIV-1 subtypes A (odds ratio, 3.8; 95% CI, 0.8-24.7%) and C (odds ratio, 5.1; 95% CI, 1.3-30.8%) were more frequently transmitted from mother-to-child than subtype D [271]. In another study, the risk of MTCT was higher in women infected with subtype C, followed by subtype A, and was lowest in women infected with subtype D. Pregnant women, infected with subtype C were more likely than those infected with subtype A or D (P=0.006) to shed HIV-1-infected vaginal cells, implying that transmission to infants or partners may be more likely with this subtype: this relationship held after adjusting for age, CD4 cell count, and plasma HIV-1 viral load [272]. Another study in Tanzania presented similar results, with preferential in-utero transmission of HIV-1 subtype C compared to HIV-1 subtype A or D (P=0.026) [273]. Other researchers, however, found no association between subtype and rates of MTCT [274-276]. In studies where pregnant women received a single-dose nevirapine (sdNVP) prophylaxis no significant differences were also observed in the rate of MTCT in women with HIV-1 subtype A, D or C [277, 278].

Many factors, such as maternal stage of the disease, maternal immunological status, viral load, mode of delivery, duration of breast-feeding, ARV prophylaxis, maternal plasma vitamin A (associated with AIDS progression), and close maternal-child Human Leukocyte Antigen (HLA) matching, can contribute to these differences [279-281]. Nevertheless, the role of virus determinants in MTCT has not been well established yet [282]. Several studies have shown that viral diversity in the mother is generally higher than that present in the infant, suggesting that maternal viruses are selected before transmission [283, 284]. Several factors like specific viral selection [285], neutralization resistance [286-288] and enhanced replicative capacity of the transmitted

viruses [289] have been associated with a bottleneck type transmission. The basis for the MTCT bottleneck is an issue that needs more clarification.

In summary, it remains to be determined whether there is a true association between subtypes and adult or MTCT transmission of HIV-1 or whether the differences in transmission probabilities found in some studies are associated with several other factors that can influence HIV transmission, e.g. behavioral, epidemiological and immunological [290]. More longitudinal and well controlled studies, preferentially performed in a single area and with a single ethnic group of HIV-1 infected patients (for instance in Angola or other country with many different subtypes), are needed to identify HIV-1 determinants for adult and vertical transmission and to evaluate the potential association of subtype with transmission.

Disease Progression

Another important question is whether clade differences result in variable rates of disease progression. There have been several prospective, observational studies of the course of HIV-related disease in cohorts infected with various HIV-1 genetic forms. Although some studies did not find an association between HIV-1 clades and disease progression [107, 291-294], more recent studies established this association [125, 295, 296]. A retrospective cohort study (1996-2007) reported that Africans patients infected with HIV-1 non-B subtypes (A, C, F-K, AC, AE, AG, BF and DF) had slower rates of disease progression compared to Haitians (P=0.0001) and Canadians (P=0.02), being both the latter groups infected with subtype B viruses [295].

Earlier studies found that subtype D was associated with the most rapid disease progression relative to other subtypes [107]. A very recent study in patients from Rakai, Uganda, reported that infection with subtype D is associated with significantly faster rates of CD4 T-cell loss than subtype A (P<0.001), which might explain the more rapid disease progression for subtype D compared with subtype A [296]. Along the same lines, a study conducted in 2010 in an ethnically diverse population of HIV-1-infected patients in South London showed a faster CD4 cell decline and higher rate of subsequent virological failure with subtype D infection than with subtypes B (P=0.02), A (P=0.004), or C (P=0.01) [125].

An important unanswered question is the biological basis for these differences. A possible clue comes from data suggesting that emergence of X4 variants, which in subtype B are associated with increased CD4 depletion and disease progression [302-304], was more common in HIV-1 subtype D compared with subtype A (P = 0.040) [266, 267]. Other study found that subtype D may be dual tropic more frequently than the other subtypes [265].The earlier switch to X4 virus with subtype D may explain the faster rate of CD4 decline and disease progression with this subtype [266, 267].

Some studies reported the presence of X4 or R5X4 isolates at early stages of infection, in addition to a decrease in CD4+ counts, in all patients infected with CRF14_BG [194]: moreover, CRF14_BG infected patients can progress very quickly to AIDS and death [297]. These results suggest that, like

HIV-1 subtype D, CRF14_BG may be highly pathogenic [298, 299]. The rapid disease progression with CRF14_BG may be due to an earlier switch to X4 phenotype driven by the selective pressure of neutralizing antibodies (see Chapter 7). However, this relationship between higher tendency for X4 use and higher disease progression may not hold for other subtypes. For instance, the percentage of X4 virus appears to be lower in subtype C than in subtype B, even when the viruses are obtained from patients with AIDS [300-302].

It is important to note that most of these studies of disease progression have confounder factors such as access to medical care, nutritional status, host genetic factors, and mode of viral transmission, which may contribute to the divergent results [303]. More studies are needed to confirm previous conflicting results, and to elucidate the host–viral interactions that may lead to more favorable outcomes in individuals infected with various genetic forms of HIV-1. This kind of studies should be longitudinal, performed with a higher number of patients, preferentially in primary infection, in a single country to better control ethnic and genetic factors of the patients, and with several genetic forms of HIV-1.

Diagnosis and Disease Management

The various measures of HIV infection have specific “detectable moments” during the natural history of infection (Figure 1.2). After infection, symptoms may appear within two weeks: in average, in the first eleven days the infection is characterized by undetectable viral markers in blood samples (window period). Plasma HIV RNA levels begin to increase at the 10^{th} day, peaking around 20 days after infection; HIV p24 levels typically peak around 20 days after infection; after that the antibody response occurs (Figure 1.2) [304]. Serological and molecular diagnostic assays have been designed to detect and/or quantify one or more of these HIV infection markers. These assays should be able to detect all genetic forms of HIV, however, the very high genetic and antigenic evolution of HIV along with the continued diversification and global redistribution of HIV groups, subtypes and recombinants may have important implications for diagnostic testing and patient management.

HIV fourth-generation assays detect both HIV antibodies and the p24 antigen. These assays provide an advantage for detection of infection during the window period prior to seroconversion since the diagnostic window may be reduced by an average of 5 days relative to an IgM-sensitive EIA [305]. However, some fourth-generation assays showed low sensitivity in the detection of p24 antigen from some non-subtype B HIV-1 strains (A, C, F, H, CRF01_AE, O) and HIV-2 [306-310]. This low sensitivity in antigen detection may be attributed to differences in viral epitopes of the different HIV genetic forms which may not be recognized by the monoclonal antibody used in the assay [311].

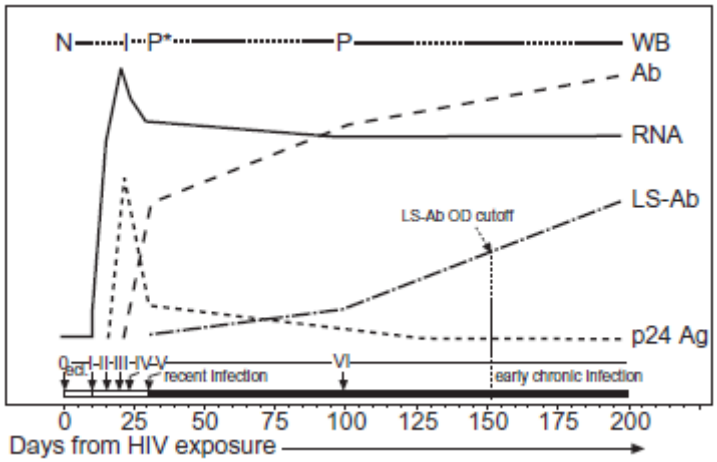

Figure 1.2 - Progression of HIV-1 markers in acute infection. WB, Western blot; Ab, HIV antibody; RNA, HIV RNA; LS-Ab, HIV antibody determined by sensitive/less sensitive enzyme immunoassay testing strategy; p24 Ag, HIV p24 antigen, from time of exposure (day 0) through the first 200 days of infection. Ecl, eclipse period (undetectable viral markers in blood samples); Stage I (definitive HIV RNA viremia), stage II (p24 antigenemia), stage III (HIV EIA antibody reactive), stage IV (I, Western blot indeterminate), stage V (Western blot positive without p31 band) and stage VI (P, Western blot positive with p31 band). Adapted from, Fiebig *et al.*, 2003 AIDS [304].

The major target for HIV-1 antibodies in immunoassays is the *env* gp41 immunodominant region (IDR). Key epitope(s) targeted by these assays might be modified or eliminated by the occurrence of natural polymorphisms within the IDR region associated with the genetic variation of HIV-1, ultimately leading to reduced sensitivity or lack of antibody detection [312, 313]. A few cases of false-negative results involving HIV-M variants (subtypes B, C, and F, for example), resulting from major mutations of the epitope in the IDR, have been described [306-308, 313-315].

Although it remains difficult to determine the frequency of false negatives for each HIV group, a large number of the reported failures involve HIV-1 group O. Earlier analysis of specimens from patients infected with group O viruses revealed that some commercial immunoassays failed to detect group O infections [316-319]. This ultimately led to incorporation of group O specific antigens and/or peptides into the assays to improve detection of group O infections [320]. Nonetheless, false-negative results continue to be reported for some patients infected with HIV-1 group O [311, 315, 321].

Despite the high genetic divergence between HIV-1 groups M and N, all group N infections studied till now were detected by five commercial HIV immunoassays [210]. Group P infections may not be efficiently detected by the current HIV screening tests due to the absence of group P-specific reagents for antibody detection [322]. Nevertheless Plantier et al., in the first report regarding detection of group P infections, found that several HIV-1 screening tests were reactive against this group [32]. Despite the absence of either HIV-1 group N and group P specific antigens in most

assays, antibodies targeting some group M specific antigens may cross-react with group N and P antigens allowing for the serologic detection of infections by HIV-1 group N and P.

Serological diagnosis of HIV-1 infection in Sub-Saharan Africa is mostly done with rapid tests [323]. This kind of assay is simple, rapid, instrument-free and relatively cheap. However some of these assays have shown problems in detecting HIV-1 subtypes D, F, H, CRF02_AG, group O and HIV-2 [314, 324-328]. Minor antigenic differences between isolates of different clades and the peptides/recombinant proteins used in these assays could explain the problems in the detection of some HIV genetic forms [314, 327-329]. Low sensitivity of these tests can also be associated with low level of HIV-specific antibodies due to recent seroconversion, early and stringent control of viral replication by antiretroviral therapy, or immune exhaustion in end-stage AIDS patients [327-333].

A variety of nucleic acid based diagnostic assays that quantify plasma HIV-1 RNA levels have been developed to monitor disease progression, response to antiviral drug therapy, detection of primary infection [plasma HIV RNA levels begin to be detectable about 11 days after infection (Figure 1.2)], early detection of HIV infection among perinatally exposed infants and HIV vaccine recipients, and detection of HIV infection in the absence of antibodies (Table 1.6)[225, 334-337]. These assays rely on HIV-1 sequence-specific primers and/or probes and use technologies such as reverse transcriptase polymerase chain reaction (RT-PCR) amplification, isothermal nucleic acid sequence-based amplification (NASBA), branched-chain DNA signal amplification (bDNA) and real-time (RT) PCR [338-345]. The genetic variation of HIV-1 presents challenges to the design of quantitative assays that measure HIV-1 RNA or DNA levels. Reliable quantification can be compromised by natural polymorphisms occurring in primer/probe sequences that have the potential to reduce or abolish hybridization [346, 347]. Genetically divergent variants may go unrecognized since, usually, subtype and target sequence information is not known at the time of testing.

Several comparative studies have shown that the sensitivity and specificity of viral load assays varies depending on HIV-1 group or subtype, especially in non-B subtypes, complex recombinant forms and groups O, N, and P viruses [32, 348-361]. However, the newer quantitative real-time PCR (qRT-PCR) methods (i.e., m2000rt Abbot Real Time HIV-1 Assay or Cobas AmpliPrep/COBAS TaqMan) showed a higher performance on HIV viral load testing of patients with subtype B as well as patients with non-B subtype infections [348-350, 352, 360]. Nonetheless, Abbot Real Time HIV-1 Assay seems to be the only assay prepared to detect all HIV-1 subtypes, several CRFs, as well as group N, and O viruses [349, 352, 360]. This is probably related with the high level of genetic conservation of the integrase gene that this test amplifies [362]. In contrast, bDNA (Versant v3.0) and NASBA (EasyQ) assays are considerably less reliable for accurate viral load measurements across HIV clades [348-350, 352, 360]. In conclusion, HIV-1 assays targeting the highly conserved *pol* integrase region of the HIV-1 genome may be subject to less variability than assays targeting the *gag* gene [352, 353, 355, 361]. As HIV genetic diversity evolves, evaluations of all commercially licensed HIV-1 viral load assays should be performed regularly in populations with patients infected with all viral subtypes.

The high costs and complexity related to plasma viral load quantification renders this method unsuitable for low-income countries that are the most affected by the HIV epidemic. Dried blood spots (DBS) are easy to collect and store, and can be a convenient alternative to plasma in settings with limited laboratory capacity [363]. The performance of DBS has been validated against plasma/serum samples with various commercially available assays and showed excellent correlation (Table 1.7). However, compared with plasma samples, the use of DBS for viral load measurement has some disadvantages which include reduced sensitivity due to small input volumes and impaired efficiency of nucleic acid extraction, as well as nucleic acid degradation under environmental conditions (review in [363, 364]). In addtion, knowing that the sensitivity and specificity of viral load assays varies depending on HIV-1 clade, probably the problems related with quantification of some HIV-1 genetic forms could be aggravated.

DBS techniques for viral load quantification should be standardized and optimized for a high-throughput setting. For low income-countries, virological assays must be simple, stable, robust, and affordable. Pooled nucleic acid amplification testing (NAAT) (or group testing) can improve efficiency and test performance of testing for primary HIV-1 infection [365]. Grouping NAAT by pooling samples together and testing the entire pool will lead to a decrease in the average number of tests performed, consequently lower costs, and may also lead to higher specificity and positive predictive values [365-367]. Currently, HIV NAAT on blood plasma pooled from blood donors is used to screen blood supplies for HIV contamination [368]. This kind of strategy could also be used to monitorize viral load in patients receiving ART treatment in developing countries since it has lower costs [369-372]. The strategy consists in pooling blood samples and performing one HIV RNA assay on a pooled sample. If the sample is negative for HIV RNA, then most likely all individuals in the pool are negative for HIV infection [365, 367, 368]. This strategy was found to be above 40% more efficient than individual viral load testing while maintaining excellent accuracy in a population with a prevalence of ART failure of 1-25% [370-372]. Furthermore the method maintained an excellent accuracy even in populations with high levels of detectable viremia [369, 372].

Failed detection or unreliable quantification of HIV infection can have significant consequences in early detection of MTCT [361, 373]. Early diagnosis can only be achieved with tests that detect HIV-1 nucleic acids such as HIV-1 DNA or HIV-1 RNA, since persistent maternal HIV antibodies in the infant until as late as 18 months [374], preclude the use of antibody detection tests. It has been recommended that diagnostic testing with HIV-1 DNA or RNA assays be performed within the first 14-21 days of life, at 1 to 2 months of age, and at 4 to 6 months of age [375]. Additionally, if any of these test results are positive, repeat testing on a second sample has to be done to confirm the diagnosis of HIV-1 infection. A diagnosis of HIV-1 infection can be made on the basis of 2 separate positive HIV-1 DNA or RNA assay results [376].

A study performed by Bogh *et al*. found that one commercial HIV-1 qualitative DNA PCR test (Amplicor HIV-1 DNA PCR test), does not detect all subtypes with equivalent sensitivity and that 10% of the positive samples tested negative [377]. These results were supported by two other reports

that found that the only commercially available HIV-1 DNA PCR lacked optimal sensitivity to detect non-B HIV-1 subtypes (Subtype C and CRF02_AG) [378, 379]. In May 2005, Roche Diagnostics replaced the version 1.0 Amplicor HIV-1 DNA PCR assay with version 1.5, which has been shown to have excellent sensitivity and specificity in testing adult venous blood samples and infant DBS [380, 381]. Amplicor HIV-1 DNA PCR version 1.5, is highly accurate in detecting the multiple HIV-1 subtypes circulating in Africa [382], is standardized and supported for use in Africa, and has been used by researchers and infant diagnosis pilot programs in several countries [373]. However, Amplicor HIV-1 DNA PCR 1.5 use primers for the relatively variable *gag* gene and was developed to amplify HIV-1 group M strains [383]. So, it is likely that sensitivity problems arise with groups O, N and P. New DNA amplification assays that cover all HIV-1 genetic forms are needed.

TABLE 1.6

Viral Load Assays Approved by FDA and Recommended by WHO

Assay	Technology	Probe target	Linear range	HIV-1 clade recognition
Abbot Real Time HIV-1	RT-PCR	*pol*-INT	40 to 10,000,000 RNA copies/mL	Group M (subtypes A-H), several CRFs and Groups O and N
Amplicor HIV-1 Monitor Test v1.5	RT-PCR	*gag*-p24	Standard: 400 to >750,000 RNA copies/mL Ultra-sensitive: 50 to >100,000 RNA copies/mL	HIV-1 Group M (subtypes A-H)
Cobas Amplicor HIV-1 Monitor Test, v1.5	RT-PCR	*gag*	Standard: 400 to >750,000 RNA copies/mL Ultrasensitive: 50 to >100,000 RNA copies/mL	HIV-1 Group M (subtypes A-H)
Cobas AmpliPrep/Cobas TaqMan HIV-1 Test, v2.0	RT-PCR	*gag*-p41 and 5´LTR	20 to 10,000,000 RNA copies/mL	Group M, several CRFs, and Group O
Versant HIV-1 RNA 1.0 Assay (kPCR)	RT-PCR	*gag*-p24	37-11,000,000 copies/mL	HIV-1 Groups M and O
Versant HIV-1 RNA 3.0 Assay	bDNA	*pol*-INT	50-500,000 copies/mL	HIV-1 Group M
NucliSens EasyQ HIV-1 v2.0	NASBA	*gag*-p24	10 to 10,000,000 RNA copies/mL	HIV-1 Group M (subtypes A-J), CRF01_AE, and CRF02_AG

Adapted from, WHO, 2010 [384].

TABLE 1.7

Recent Studies Comparing HIV-1 Viral Load in DBS with a Gold Standard Plasma Method

Viral Load Assay	Extraction Method	Lower Detection Limit*	Ref
COBAS TaqMan RT-PCR Assay	Nuclisens MiniMAG	3.0 (96.4% detected)	[385]
COBAS TaqMan RT-PCR Assay	Primagen	3.0 (96 % detected)	[386]
Nuclisens EasyQ HIV-1 v2.0	Nuclisens EasyMAG	2.9 (95% detected)	[387]
Nuclisens EasyQ HIV-1 v2.0	Manual Nuclisens	3.48 (100% detected)	[388]
Nuclisens EasyQ HIV-1	Nuclisens MiniMAG	2.9 (100% detected)	[389]
Amplicor HIV-1 Monitor Test v1.5	In-house method	3.0 (100% detected)	[390]
Abbot Real Time HIV-1	m2000 RT system	3.72(100% detected)	[391]
Abbot Real Time HIV-1	m2000 RT system	2.6 (99% detected)	[392]
Abbot Real Time HIV-1	m2000 RT system	3.0 (100% detected)	[393]

*Correlation with plasma viral load results, expressed as Pearson's or Spearman's correlation coefficient or R2 as appropriate. Adapted from, Johannessen, Bioanalysis 2010 [363].

Vaccination

Ultimate control of the HIV-1 pandemic is dependent on the development of an effective preventive vaccine [394, 395]. The design of such vaccine must consider all the diversity observed in cellular and neutralizing epitopes [396]. The enormous genetic diversity and other unique features of the HIV envelope (Env) protein [397-400] make this a difficult task, however the 30% of protection seen in a recent efficacy trial in Thailand (RV144) [401] indicate that the development of a vaccine is possible.

Hosts infected with HIV-1 have cellular and humoral immune responses to their infecting strains, but there is evidence of mutational escape of viruses from responses by CD8+ cytotoxic T cells and neutralizing antibodies (NAb) over time [402-406]. The question whether T cells are capable of cross recognizing genetically diverse virus isolates representing multiple subtypes is the key for HIV vaccine development and has been addressed in several studies [407-417]. Although cross reactive responses to other viral subtypes have been shown [411, 415-417], the strength and breadth of these responses were typically limited [414]. Recent studies showed that increased breadth and depth of these responses could be achieved with mosaic immunogens [416, 417]. Nonetheless although these types of vaccines recognize diverse forms of an HIV-1 epitope, they do not confer sufficient protection against different genetic forms of HIV-1 [418]. As a consequence, a fully effective, preventive vaccine regimen will probably need to induce strong, cross-subtype HIV-specific T-cell immunity as well as broadly reactive NAb activity to overcome the challenge of HIV diversity [394, 419].

Development of a vaccine that induces NAbs that bind to Env remains a great challenge (review in [419]). Most monoclonal antibodies (mAbs) against HIV-1 Env have been derived from subtype B-

infected patients (review in [419, 420]). Numerous mAbs to the HIV-1 Env have been isolated from humans, but only a few of these can effectively neutralize most strains of the virus (review in [419, 420]). This highlights one of the central features of the humoral immune response against HIV-1: most of the anti-Env antibodies generated during natural infection are directed to regions of gp120 or gp41 that are not exposed on the mature functional virus spike. However, sera from some HIV-1-infected subjects are able to potently neutralize diverse isolates of HIV-1 [419], which demonstrates that there are vulnerable regions on the functional Env trimer. b12 was the first broadly reactive, neutralizing human mAb isolated from a patient infected with a subtype B virus [421]. This mAb can neutralize more than 50% of subtype B viruses and about 30% of non–B viruses (A, C, D, CRF01_AE and CRF14_BG) [422, 423]. Other human anti-CD4 binding site (CD4bs) mAbs have been isolated, but most of these are considered to be non-neutralizing or are only able to neutralize a limited subset of highly sensitive tier 1 strains of HIV-1 [422, 425] (viruses are classified as tier 1 when they are highly susceptible to neutralization, and as tier 2 when they show a lower level of neutralization sensitivity [424]). For instance, mAb 2G12 has quite limited activity against non-B subtype viruses and, like mAb 2F5 (anti-MPER mAb), it is especially weak against subtypes A and C strains (reviewed in [419, 420]).

Some studies found broadly reactive antibodies from serum of some HIV-1-infected patients that can neutralize several HIV-1 strains including viruses from different subtypes (review in [419, 420]). This broadly reactive NAb response was initially thought to be quite uncommon, but some studies show that approximately 25% of HIV-1 positive sera can neutralize many circulating viruses, and a subset of these sera, perhaps 10%, contains NAbs that can neutralize most known HIV-1 strains (review in [419, 420]). Several highly selected sera have been shown to contain broadly NAbs to the CD4bs, and this has been demonstrated for both subtype B and C HIV-1-positive sera (review in [419, 420]), which proves that NAbs to a highly conserved region of HIV-1 can be generated. However, anti-CD4bs antibodies do not appear to constitute the major serum neutralizing fraction in most cases (review in [419, 420]). Some sera can neutralize diverse HIV-1 strains by gp120-directed antibodies that are not targeted to the CD4bs, while other sera contain NAbs not directed to monomeric gp120 at all, and hence may bind to epitopes formed by the native trimeric configuration of the HIV-1 Env (review in [419, 420]). Further investigation and improved epitope mapping technologies will be required to understand the full spectrum of the NAb response against the different HIV-1 genetic forms.

Studies have suggested that clade-specific differences may be related with different neutralizing capacity. Several studies found that viruses with shorter V1–V4 loops induced antibodies with more neutralization breadth against subtype C viruses [399, 426, 427]. Subtype C is more variable in the C3 region, particularly in the α2-helix, which is relatively conserved in subtype B [398, 428]. Another study that compared sequence and structural characteristics of gp120 domains, that are under distinct selection pressures in subtypes B and C revealed subtype-specific patterns of variation with structural and antigenic implications [399]. They found that in subtype C, the C3

region α2-helix exhibits high sequence entropy at the polar face while maintaining its amphipathicity, whereas in subtype B it accommodates hydrophobic residues. In addition, it was found that the V4 hypervariable domain in subtype C is shorter than that in subtype B [399]. Codon-specific ratios of nonsynonymous to synonymous substitution rates (dN/dS; where a high ratio is indicative of positive or diversifying selection) are dramatically different in the subtypes B and C V3 and C3 regions of gp120 [398, 428]. The V3 loop from subtype B has a high density of states with dN/dS >1, whereas those from subtype C show little variation [429].

Important differences in the evolutionary rates of the different subtypes that reflect their interplay with the immune system and may have an impact on vaccine development and vaccine response have been found [430]. These differences are caused by both different selective pressures (for dN rate) and the replication dynamics (for dS rate) (i.e., mutation rate or generation time) of the strains. CRF02_AG and subtype G have higher mutation rates, while subtype D has lower dN and dS rates when compared to other subtypes. In a comparison of the V3 loop (35 aminoacids) to the flanking regions of the V3 loop, D subtype viruses were found to have lower dS/dN ratios (the greater the dS/dN ratio, the greater the relative conservation) in the V3 loop. This suggests a greater rate of change at the protein level within the D subtype V3 loops than in the flanking regions. All other subtypes had higher dS/dN ratios in the V3 loop suggesting that for the other subtypes V3 loops were more conserved than V3 loop flanking regions [431]. These data may implicate an increased difficulty in finding a vaccine against subtype D viruses. The immunological impact of these differences is not well understood, but these features should be considered when selecting vaccine antigens.

Several candidate HIV-1 Env-containing vaccines have been evaluated in human clinical trials (reviewed in [419]), most of the immunogens used in these vaccines beeing from HIV-1 subtype B. However an effective vaccine must incorporate all genetic forms of HIV. The ongoing and planned clinical trials of HIV-1 vaccines are described in Table 1.8.

One heterologous prime-boost vaccine has been tested in a recent efficacy trial. This trial (RV144), which was conducted in Thailand and completed in October 2009, enrolled 16,402 individuals, mainly heterosexuals, at risk of HIV-1 infection [401]. A recombinant canarypox vector, ALVAC-HIV (vCP1521) was one of the components of the vaccine. This vector expressed HIV-1 Env (from CRF01_AE 92TH023 and subtype B LAI strains), Gag (subtype B LAI strain) and PR (subtype B LAI strain) sequences. The second component was a recombinant glycoprotein 120 subunit vaccine named AIDSVAX B/E. These protein sequences derived from CRF01_AE A244 and subtype B MN strains. Vaccination appeared to reduce in 31.2% the rate of acquisition of HIV- 1 infection but had no significant effect on early viral loads or on CD4+ T cell counts in vaccine recipients who later acquired infection. The modest protection seen in the RV144 trial [401] may indicate that weak NAb responses and/or other antiviral antibody effector mechanisms affording protection against HIV-1 infection have greater value than previously thought. Although it is not yet known what role antibodies played in this protection, it remains likely that anti-Env antibody responses will be a

critical component of an optimally effective HIV-1 vaccine. Thus, additional improvements in immunogen design are needed to achieve a more acceptable level of antibody mediated protection against all HIV genetic forms including HIV-2.

TABLE 1.8

Ongoing and Planned Clinical Trials with HIV-1 Env Immunogens

Env immunogen[a]	HIV-1 strain	Subtype
VEEgp120	Du151	C
DNA-polyvalent gp140ΔCFI prime/Ad5-polyvalent gp140ΔCFI boost	Bal/IIIB chimera	B
	92RW020	A
	97ZA012	C
DNA-gp140 prime, MVA-gp140 boost	ADA	B
DNA-gp140 prime, MVA-gp140 boost	Du151	C
DNA-gp140, DNA-IL-12 or DNA-IL-15	Consensus sequence	B
Ad35-gp140 prime/Ad5-gp140 boost	92RW020	A
Ad5-gp140 prime/Ad35-gp140 boost	92RW020	A

[a] Unless indicated otherwise (e.g., delivery via recombinant DNA or viral vector), Env immunogens were recombinant proteins. VEE, Venezuelan equine encephalitis virus; Ad5 and Ad35, adenovirus serotypes 5 and 35, respectively; MVA, modified vaccina Ankara (attenuated strain of vaccinia virus). Some vaccines contained additional HIV-1 immunogens that are not shown because they are not targets for NAbs; these immunogens were incorporated into vaccines for elicitation of virus-specific T cell responses.

In conclusion, the results of the Thailandese trial (RV144) have brought a new optimism and reassure that the development of an effective HIV vaccine is possible. In addition researchers are becoming more conscious of the need to incorporate several HIV-1 genetic forms in vaccine candidates due to the impact of genetic diversity in immune response and consequently in vaccine design.

Response to Antiretroviral Therapy

Differences in amino acid composition between HIV-1 clades can lead to differences in susceptibility to ARV drugs. This is best illustrated by HIV-1 group O and HIV-2 isolates that show high-level of innate resistance to NNRTIs and T20 [432-434]. This innate resistance is due to resistance mutations that are present as natural polymorphisms. For instance, HIV-1 group O isolates naturally present a cysteine at RT position 181 (Y181C) which is considered a major DRM to NNRTIs; the secondary NNRTI DRM A98G is also a natural polymorphism in group O [432, 433] (Table 1.9).

TABLE 1.9

Polymorphisms of HIV that May Have Impact on Emergency of Resistance to NRTIs and NNRTIs

Polymorphisms	Drug Class	% in subtype B	% in non-B subtypes
A98S	NNRTIs	5%	70% G and 98% O
K103R	NNRTIs	2.7%	98% O
V106I	NNRTIs	1.7%	100% HIV-2
V179E	NNRTIs	0.4%	98% O
V179I	NNRTIs	3.2%	>90% HIV-2 and 50% A
Y181C	NNRTIs	0%	100% O

Adapted from, Wainberg *et al.,* Viruses 2010 [435].

Susceptibility of non-B subtypes to ARV drugs has been less well studied than subtype B mainly because of the predominance of subtype B in developed countries where ARVs first became available, coupled with the availability of genotypic and phenotypic ARV drug resistance testing [436]. Some studies of sdNVP for prevention of MTCT have demonstrated a statistically significant disparity in the overall drug resistance among subtypes, with frequencies of 69-87%, 55.3-36%, 19-42%, and 21% resistance against NVP in women with subtype C, D, A, and CRF02_AG infections, respectively [437-440]. There were no significant differences in the pre-NVP frequency of NVP resistance mutations or the pre-NVP levels of K103N-containing variants in women with subtypes A, C, and D that could explain the subtype-based differences in mutations after sdNVP exposure [438]. However there are other factors that may be associated with NVP resistance in women after the administration of sdNVP and which include: higher viral load and lower CD4+ cell count prior to NVP exposure, increased pharmacokinetic exposure to NVP (e.g., longer half-life and decreased oral clearance of NVP), and the timing of sample collection [437]. Additional studies are needed that take in account all these factors to better understand the biological causes of these subtype differences in sdNVP resistance.

In the Pediatric European Network for Treatment of AIDS (PENTA) 5 trial, where 128 children were enrolled in a randomised trial to evaluate the antiviral effect of NRTI combinations (3TC+Abacavir, 3TC+ZDV, Abacavir+ZDV) and the tolerability of adding NFV, there was no significant difference according to HIV-1 subtype, in the virologic response to treatment or in the frequency of development of resistance among children [441].

A French cohort study of 416 adult patients, 24% of whom carried HIV-1non-B subtypes, showed that at 3, 6, and 12 months after initiation of ARV therapy (first line regimens, subtype B: 65% PI-based regimens, 25% NNRTI-based regimens, 10% NRTI only; non-B subtype: 65% PI-based regimens, 30% NNRTI-based regimens, 5% NRTI only), HIV-1 subtype did not affect clinical progression, CD4 cell count, or viral load in response to treatment [442]. Frater *et al*. studied patients of African origin who were infected with a non-B subtype of HIV-1 and were living in London, and found no significant difference in the response to therapy (first line regimens, 50% PI-based regimens, 50%

NNRTI-based regimens) among patients infected with subtype A, C, and D [443]. Geretti and collaborators reported that patients infected with prevalent non-B subtypes (A, C, D and CRF02_AG) were as likely to achieve viral load suppression (NRTI backbone: AZT+3TC or TDF+FTC or TDF+3TC or 3TC+d4T or d4T+ddI or ABC+3TC; third drug: EFV or NVP or RTV boosted PI) as those infected with subtype B and showed comparable rates of CD4 cell count recovery [444]. Other studies have analyzed virologic and immunologic responses to antiretroviral therapy according to the HIV-1 subtype and also did not find any differences [445-449]. Thus, overall, it appears that HIV-1 subtypes do not have major differences in the response to ARV therapy. However, further studies should be designed, firstly to assess the efficacy of specific drug regimens in patients with non-B subtypes, and secondly, to evaluate the efficacy of these regimens in patients with particular non-B subtype species including the highly divergent H, J and K subtypes, complex CRFs and URFs and groups O, N and P viruses, which are common in African countries. These studies should be performed in a single country in order to control for the many variables that might influence response to therapy, namely, adherence, ethnicity, psychosocial support and drug regimens.

Resistance to Antiretroviral Therapy

In the absence of any drug exposure, RT and PR sequences from B and non-B subtypes are polymorphic in about 40% of the first 240 RT amino acids and 30% of the 99 PR amino acids [148, 256, 450]. Polymorphisms in the RT of non-B subtype viruses normally do not occur in known sites of resistance to NRTIs [451]; in contrast, the PR from drug naive patients may contain amino acid substitutions associated with secondary resistance to some PIs in subtype B (ex. K20R, M36I, H69KQ) (Table 1.10) [452, 453]. However, these genotypic changes by themselves do not consistently confer decreased susceptibility to PIs when viral strains are subject to phenotypic testing [160, 252, 454-462]. Consistent with this, most observational studies performed *in vitro* and *in vivo* suggest that the currently available PR and RT inhibitors are as active against non-B subtype viruses as they are against subtype B viruses [106].

Different HIV genetic forms carry in their genomes genetic signatures and polymorphisms that could alter the structure of viral proteins which are targeted by drugs, thus impairing ARV drug binding and efficacy (Table 1.9 and 1.10). A single nucleotide substitution from the wild-type codon found in subtype C can generate the mutation V106M, which is associated with NNRTIs resistance, while at least two substitutions are needed for the wild-type subtype B codon [463, 464]. This suggested that subtype C could have a lower genetic barrier to resistance to NNRTIs than subtype B, and that this V106M mutation could be more frequent in subtype C infected patients failing therapy, than in subtype B infected patients. Indeed, the clinical importance of the V106M mutation in non-B subtypes has been confirmed in several studies showing that V106M is more frequently seen in subtype C (and CRF01_AE) after therapy with EFV or NVP [465-468]. The G190A mutation was also

relatively more frequent in subtype C Indian and Israeli patients failling NNRTI-based regimens than in subtype B [461, 465].

In vitro, the emergence of the K65R mutation after therapy with TDF is faster in subtype C (15 weeks after TDF) than in subtype B (34-74 weeks) [469-471]. In contrast, K65R may be less frequent in subtype A than in all other subtypes [472]. Clinically, K65R has been seen in approximately 70% of patients failing ddI and d4T containing regimens in Botswana [473], and in Malawi in patients with subtype C viruses [474]. A study from Israel reported a high frequency of K65R in subtype C viruses from Ethiopian immigrants in ARV therapy [475]. However, K65R did not appear to emerge frequently in subtype C patients who participated in large clinical trials in which they received either TDF or TDF/FTC as part of a triple therapy regimen [476]. In Malawi, in patients with subtype C viruses, differences observed in the emergence of the K65R mutation were significantly related to treatment regimen and disease stage [474]. In addition, development of K65R in subtype C and CRF01_AE has been associated with the Y181C NVP mutation within the viral backbone [477, 478]. The presence of higher rates of the K65R mutation in subtype C in some studies [473, 474, 479] suggests that these viruses may have a particular predisposition toward acquiring this mutation. It has been proposed that a RNA template mechanism could explain the higher rates of K65R in subtype C viruses than in other subtypes. In this subtype, there is an intrinsic difficulty in synthesizing pol-A homopolymeric sequences that leads to template pausing at codon 65, facilitating the acquisition of K65R under selective drug pressure [469, 470]. Several studies also suggest that there is a higher risk of development of K65R in subtype C infected patients failing ddI and d4T-containing regimens [473, 477, 479, 480]. The presence of the mutation Y181C was also associated with development of K65R in subtype C [477].

The natural polymorphisms found in the RT of treatment-naive patients (10% in 726 patients) infected with HIV-1 non-B subtypes had no significant impact on susceptibility to ETR [481-483].

In PR, polymorphisms do not impair drug susceptibility but may affect the genetic pathway of resistance as soon as the virus generates a major resistant mutation [484]. The rare minor V11I mutation, which is associated with DRV resistance, is a natural polymorphism in all CRF37_cpx isolates and some subtype A isolates [60, 148, 256, 485, 486], suggesting that these viruses may have a lower genetic barrier to DRV resistance. The V82I natural polymorphism in subtype G led to the emergence of I82M/T/S with treatment failure to IDV [487]. A study suggested that polymorphisms at position 36 in PR may play important roles in determining the emergence of specific patterns of resistance mutations among viruses of different subtypes [488]. Gonzáles *et al*. compared clinical isolates of C subtype with and without the I93L polymorphism, finding that hipersusceptibility to LPV in subtype C is strongly associated with the presence of that mutation [489].

TABLE 1.10

Polymorphisms of HIV-1 Non-B Subtypes Associated with Resistance to PIs

Minor mutations	ARV	% in subtype B	Polymorphisms
V11I	DRV	1%	100% CRF37_cpx and 4% in subtype A
I13V	TPV	13%	90%–98% in subtypes A, G and CRF02_AG, 4%–78% in other non-B subtypes
K20I	ATV	2%	93%–98% in subtypes G and CRF02_AG, 1%–3.5% in subtypes A, F and CRF01_AE
M36I	ATV, IDV, NFV and TPV	13%	81%–99% in several non-B subtypes
H69K	TPV	2%	96%–97% in subtypes A, C and G, CRF01_AE and CRF02_AG, 2% in subtype F
V82I	ATV	2%	87% in subtype G, 1%–6% in several non-B subtypes
I93L	ATV	33%	94% in subtype C, 5%–40% in several non-B subtypes

DRV, darunavir; TPV, tripanavir; ATV, atazanavir; IDV, indinavir; NFV, nelfinavir.

Adapted from Santos *et al.*, Viruses 2010 [106].

The D30N mutation was not observed in CRF02_AG and CRF02_AE isolates from patients failing NFV therapy; rather, the N88S mutation emerged after NFV use in CRF01_AE and after IDV use in subtype B [490, 491]. The M89I/V mutations have been observed in F, G and C subtypes in PI experienced patients (NFV, APV, IDV, LPV, ATV) but not in other subtypes [492]. The L90M mutation, that confers resistance to NFV and SQV, is rare in subtype F but common in subtype B in patients from Brazil [493]. Another study found that D30N has a stronger negative impact in the replicative capacity of C subtype than in B subtype [494], which could explain the low frequency of this mutation observed in subtype C infected individuals failing NFV-containing regimens. A recent study in Portuguese patients, reported that mutation I54V/L was selected by NFV in subtype G isolates, a mutation not previously described for this drug in subtype B [495].

A study on polymorphisms in gp41 among different HIV-1 clades from T20 drug-naive patients found that the frequency of polymorphisms was higher in non-B subtypes and recombinants than in subtype B viruses ($p<0.001$) [496]. Almost 60% of the samples had polymorphisms at positions N42S, Q32L/T/N/K, R46K/Q, N43H, I37L, and V69L associated with resistance to T20. The N42S polymorphism, associated with increased susceptibility to T20, was detected more frequently in non-B subtypes than in B subtype(13% in B, 73% in A and 90% in G). Other study found that A30V (subtype G and CRF06_cpx) and Q56K/R (subtypes A and J, CRF04_cpx, CRF09_cpx, CRF11_cpx, and CRF13_cpx) in group M, Q56R and S138A in group O, and S138A in group N are natural polymorphisms associated with T20 resistance [497].

Several studies that analyzed drug-naive and ARV-treated patients infected with HIV-1 subtype B (all integrase inhibitor naive) found that integrase inhibitor-associated mutations (primary and secondary) are normally absent [498, 499]. A study found integrase gene polymorphisms present in more than 10% of the 97 analyzed sequences (subtype B and non-B) from patients treated with RAL, however these polymorphisms showed no impact on virological outcome either at week 24 or at week 48 ($P>0.002$) [500]. N155H, Q148H/R/K with G140S/A, and Y143R/C are the described mutational patterns that confer resistance to RAL, with or without secondary mutations [501]. A study on natural polymorphisms and mutations associated with resistance to integrase inhibitor in drug-naive and ARV-treated patients (all integrase inhibitor naive) found that CRF02_AG and subtype C isolates could have a higher genetic barrier to the development of G140C or G140S compared to subtype B [502]. On group O viruses natural presence of the E157Q mutation or E157E/Q mixture seems to confer resistance to RAL [503].

These observations in non-B subtype viruses suggest that differences in drug resistance pathways between HIV-1 subtypes do exist. However, the accumulated evidence is insufficient to adequately assess the contribution of the innate genetic diversity of HIV-1 to resistance. Larger and more rigorous prospective studies in drug naive and treated patients are required to validate these hypotheses and it will be necessary to evaluate these mutations by the analysis of site-directed mutants in phenotypic resistance assays.

Performance of Genotypic and Phenotypic Drug Resistance Assays

Drug resistance testing is extremely important for the management of ART therapy failure in HIV patients [504-506]. Genotypic and phenotypic assays are both used to detect resistance to ARV drugs that could compromise treatment response [507]. All current clinically used genotypic assays involve sequencing the genes whose proteins are targeted by the ARVs (*pol* (RT, PR and IN) and *env),* to detect mutations that are known to confer phenotypic drug resistance. There are two approved genotyping resistance assays commercially available, the ViroSeq HIV-1 genotyping system, version 2.0 [508] and the Trugene HIV-1 genotyping kit for drug resistance [509]. Phenotypic assays measure the ability of an HIV-1 isolate to grow *in vitro* in the presence of an inhibitor, in comparison with a known susceptible strain.

The European HIV Drug Resistance Guidelines Panel recommends genotyping in most situations, using updated and clinically evaluated interpretation systems [507]. The genotypic assays are faster and cheaper than phenotypic assays [510]. Despite these advantages, the commercial genotypic tests are too expensive to be used in low-income countries. In-house methods for genotyping drug resistance mutations are recommended by WHO for surveillance of primary and secondary drug resistance [511]. The reported rate of success in amplification and sequencing with these methods, for seven low-income countries, was 41-100 % in non-B subtypes (review in [511]).

Several studies analyzed the performance of commercially available genotypic resistance assays and in-house methods in B and non-B strains [512-516]. In commercial kits a greater degree of success was obtained when sequencing subtype B isolates compared to non-B isolates, and some studies report that alternative amplification/sequencing primers had to be used for some samples belonging to non-B subtypes. A Belgian study analyzed the performance of the ViroSeq HIV-1 Genotyping System in 383 samples comprising 12 different subtypes [516]. Amplification failed in 8.4% of the samples and there was a lower performance in the amplification of non-B subtypes. The sequencing performance on the different subtypes showed a significant decrease of positive results for subtypes A, G and recombinant strains. As a result of sequencing problems, 18.5% of the samples had to be processed with in-house procedures.

In Cameroon, where all groups of HIV-1 circulate, the sequencing efficiency of the ViroSeq assay was also evaluated [577]. The sequencing failures involved mainly the 5´end of the PR and the 3` end of the RT genes because of the high failure rate of primers A, D, F, and H. There was a high degree of polymorphism in non-B isolates in the areas for which these primers are designed, compared to subtype B strains [512]. One study compared two commercially available sequencing kits with a in-house genotyping system in HIV-1 samples from treated and untreated patients belonging to subtypes A through J [514]. All the samples could be amplified and sequenced by the three systems; however, for all systems, alternative amplification/sequencing primers had to be used for some samples belonging to non-B subtypes.

Several studies have evaluated the use of DBS for HIV-1 genotypic resistance testing (review in [363]); most report a high concordance between nucleotide sequences derived from DBS and plasma. Nucleotide similarity between the two sample types ranged from 98.1 to 99.9%. Drug-resistant mutations found in plasma were detected in 82–100% of the corresponding DBS specimens.

The findings of these reports indicated that the performance of amplification and sequencing primers must be improved to allow good sequencing results and consequently fast and reliable resistance testing for all HIV-1 genetic forms. Validated in-house methods with primers designed on the basis of the local HIV genetic diversity are needed for low–resources settings.

Drug resistance interpretation algorithms are user friendly and helpful in the clinical setting to follow up of HIV-infected patients. These algorithms have been developed to interpret complex patterns of resistance mutations in HIV-1 subtype B. The most frequently used clinically available systems are listed in Table 1.11. There are two types of systems: geno2pheno and *Virtual*Phenotype which try to predict viral phenotype under the assumption that phenotype predicts treatment response, whereas all others algorithms try primarily to predict treatment response, based on information extracted from databases of genotypic and correlated phenotypic or treatment response data [507].

TABLE 1.11

Drug Resistance Interpretation Algorithms

System	Levels of Resistance	Web Site
HIV DB Stanford	S, PL, LL, IR, HR	http://hivdb.stanford.edu/
REGA	S, I, R	http://www.kuleuven.ac.be/rega/cev/links/
ANRS	S, I, R	http://www.hivfrenchresistance.org/index.html
GenoSure	S, RP, R	http://www.monogramhiv.com
ResRis	S, I, R	http://www.retic-ris.net
HIVGrade	S, I, LS, R	http://www.hiv-grade.de
AntiRetroScan	100/75/50/25/0[#]	http://www.hivarca.net/includeGenpub/AntiRetroScan.html
HIV-TRePS	Quantitative*	http://www.eurist.org
EuResist Network	Quantitative*	http://www.eurist.org
Geno2pheno	Quantitative, S, I, R	http://www.geno2pheno.org
Virco	Quantitative[+]	http://www.vircolab.com
ViroSeq	S, P, R	http://www.abbotmolecular.com
TruGene	S, I, R	http://www.labnews.com

S, susceptible; PL, possible low-level resistance; LL, low-level resistance; IR or I, intermediate resistance; HR, high level resistance; R, resistance; RP or P, resistance possible; LS, low susceptibility; [#]100/75/50/25/0 in %activity with drug-GSS weighting factor; *probability for short-term response with specific drug combinations; [+]lower clinical cut-off at 20% of loss of response, upper to 80%. Adapted from, Vandamme *et al.*, 2011 AIDS Rev [507].

Several studies have compared these algorithms in drug naive and treated patients infected with non-B subtypes to examine the influence of pre-existing polymorphisms on predictions of drug susceptibilities and the subsequent choice of therapy [517-522]. Most of these studies found some discordance between algorithms, which was related to the presence of naturally occurring polymorphisms in non-B subtypes [517-522].

A study showed that, according to available resistance algorithms, both B and non-B subtypes from drug naive patients (180) were considered fully susceptible to PIs, except for TPV/RTV for which the ANRS algorithm scored non-B subtypes as naturally resistant [517]. The discordant results for TPV/RTV were obviously due to differences in the mutations that are considered by the algorithms in the analysis. The ANRS algorithm takes in account TPV/RTV mutations that are considered natural polymorphisms in non-B subtypes (e.g. M36I, H69K and L89M). In another study, 68 drug naive and 9 highly ARV-experienced HIV-1 group O infected patients were analyzed [518]. Twelve minor resistance mutations, present in more than 75% of the PR sequences, led to the different algorithms giving discrepant results for NFV and SQV susceptibility.

A large study (5030 patients infected with different HIV-1 clades) found that the four algorithms analyzed agreed well on the level of resistance scored, and that the discordances could be attributed to specific (subtype-dependent) combinations of mutations [519]. In a comparison of five algorithms in HIV-1 sequences from drug naive patients (354), discordances were significantly higher in non-B vs. B variants for ddI, NVP, TPV, and fAPV, and were attributed to natural patterns of mutations in non-B subtypes [523]. Several other studies demonstrated that there was a lack of concordance between algorithms that predict treatment response based on phenotype and genotype [495, 524, 525]. These discrepancies indicate that the patterns of drug resistance mutations have not yet been completely clarified in non-B subtype variants. The use of certain algorithms could lead to an overestimation of the resistance in the analysis of specific non-B subtypes because of the lack of consensus in the resistance mutations considered, although with increasing knowledge such discrepancies are diminishing. Nevertheless, these limitations do not imply that the analyzed tools are useless, or that the current interpretation algorithms may be invalidated due to non-B subtypes.

Tropism testing is recommended before the use of a CCR5 antagonist drug [526]. In general, the enhanced sensitivity Trofile (ESTA) assay (phenotypic assay) and V3 population genotyping are the recommended methods. A multicenter prospective study evaluated the performance of genotypic algorithms for prediction of HIV-1 coreceptor usage in comparison with a phenotypic assay for the determination of coreceptor usage [527]. Researchers reported important differences between 13 algorithms in the sensitivity of detection of X4 isolates. The most sensitive were PSSM and Geno2pheno, with sensitivities of about 60%; on the other hand the specificity was high for most algorithms. In other studies, higher sensitivities could be found for the same genotypic algorithms [528, 529]. Geno2pheno presented sensitivities of 88-93.7% and specificity of 87%, and PSSM with sensitivities of 77% and specificity of 94%. Overall, these studies validate genotypic algorithms for prediction of HIV-1 coreceptor use in antiretroviral-experienced patients infected with subtype B.

Few studies have evaluated the performance of genotypic algorithms for prediction of HIV-1 coreceptor use in non-B subtype viruses. An initial report showed a poor performance of genotypic tools for non-B subtypes (A-J, CRF01_AE, CRF02_AG, CRF11, CRF12_BF, CRF14_BG, URFs, and U samples), where they particularly failed to detect X4 strains [530]. Other studies found that main genotypic algorithms perform well when applied to CRF02_AG [531] and subtype C viruses [532]. Additional studies will be needed to further study the prediction of coreceptor use in other HIV-1 non-B subtypes.

Aim of the Studies and Work Plan

HIV-1 infection is a major public health problem, particularly in Sub-Saharan Africa. The diagnosis, therapy and prevention of this infection depend on a thorough knowledge of its epidemiology and its agents at the genetic, antigenic and phenotypic levels. As reviewed above, different HIV-1 genetic forms may have different impacts on diagnosis, transmission, disease progression, management of disease, response to ARV therapy and emergence of DRM. However, as most of the studies on HIV-1 molecular epidemiology and drug resistance have been performed in developed countries (mainly with subtype B) there is a knowledge gap in non-B subtypes and other highly divergent genetic forms (complex recombinants and URFs in group M as well as group O, N and P viruses). The recent introduction of ARV therapy in developing countries (Africa and Asia) and the wider dissemination of accessory technologies (e.g. viral load assays and CD4 cell counting equipment) has provided an opportunity to better investigate the impact of HIV-1 diversity in all of the issues mentioned above. In particular, it is important to monitorize the nature and dynamics of emergence of DRM in naive and treated HIV-1 patients as this will guide the optimal use of ARV drugs and prevent transmission of resistant strains, thereby maximizing the long term efficacy of ARV therapy in these countries.

The studies on the molecular epidemiology and drug resistance of HIV-1 described in this thesis were performed in Angola, Mozambique, Cape Verde and Portugal, countries that have strong historical, social, cultural and economical linkages between them. HIV-1 affects these countries in very different ways; whereas in Mozambique the HIV-1 epidemic already affects more than 11.5% of the population, with some Southern provinces reaching 25.1%, Angola has a moderate prevalence of 6.5%, with Luanda, the Capital city, having the highest prevalence in the country (13.6%). Cape Verde has a very low HIV-1 prevalence for an African country, not exceeding 1%. In Portugal, HIV-1 prevalence is low when compared with these countries (0.6%) but, in fact, it has the second highest prevalence in the European Union (only surpassed by Estonia). A second aspect that differentiates the HIV epidemics in these countries is the main viral transmission routes: in Mozambique, Angola and Cape Verde HIV is almost exclusively transmitted through heterosexual contact, whereas in Portugal transmission by intravenous drug use accounts for 39.9% of the HIV infections. A third potential factor of differentiation of the HIV-1 epidemics in these countries is the genetic characteristics of the infecting variants. Whereas subtypes B and G and B/G recombinants are the major genetic forms circulating in Portugal, little is known about the genetic diversity of HIV-1 in Angola, Mozambique and Cape Verde. Moreover, little is known about the origin and epidemiological history of the different HIV-1 subtypes and CRFs in these countries, as well as their potential impact in diagnosis, susceptibility to ARV therapy and drug resistance. Finally, little is known about the nature and prevalence of ARV DRM (primary and secondary) in these African countries.

The aims of this thesis were to better characterize the HIV-1 diversity in Portugal, Angola, Mozambique and Cape Verde and investigate the origin and epidemiological history of HIV-1 in these countries. The impact of these issues (genetic diversity and molecular epidemiology) in diagnosis, disease progression and susceptibility to ARV therapy was also investigated. Finally, the nature,

dynamics and prevalence of primary (transmitted) DRM was determined in untreated HIV-1 infected patients from Angola, Mozambique and Cape Verde.

Epidemiologic, clinic and virologic information as well as plasma samples were collected between 1993 and 2007 from untreated and/or treated HIV-1 infected patients from Angola (all provinces but mostly Luanda) (Chapters 2 and 3), Mozambique (Maputo) (Chapter 4) and Cape Verde (Santiago Island) (Chapter 5). For subtyping and/or resistance mutation analysis, DNA sequences were obtained from the *gag* (p17), *pol* (PR and RT) and/or *env* (C2V3C3) genes after PCR amplification using an in-house protocol. Viral genotypes were determined by phylogenetic analyses using genomic reference sequences collected worldwide and deposited in the Los Alamos Sequence Database. DRM in the *pol* gene of HIV-1 isolates from treated and untreated individuals were investigated by genotypic analysis using the Stanford Genotypic Resistance Interpretation Algorithm (adjusted for primary and secondary drug resistance mutations). To define if a mutation was a natural polymorphism of a specific subtype, the frequencies of all polymorphisms found in the sequences from treated and untreated HIV-1 patients were compared with worldwide sequences of the same subtype and/or with subtype B sequences. In untreated patients that harboured resistant viruses, the resistance profile was analyzed to predict the most effective first line regimen. In treated patients with drug resistant viruses we tried to correlate the presence of DRM with immunological and virological parameters and with the ARV regimen in use. Phylogenetic methods were used to set up the origin of HIV-1 resistant strains and to see if the samples were epidemiologically related (Chapter 3, 4 and 5).

In Angola, the perceived high HIV-1 genetic diversity and the geographical proximity to Democratic Republic of Congo (former Zaire), one of the epicenters of the HIV-1 epidemic, led us to investigate the hypothesis that Angola was also at the epicenter of the HIV-1 epidemic (Chapter 2). In Maputo, Mozambique, our main questions in the issue of HIV diversity was whether subtype C was the prevailing subtype as found in South Africa, and where did it come from (South Africa, India?) (Chapter 4). Both in Angola and Mozambique we were also interested in characterizing the natural polymorphisms in the *pol* gene that could have an impact on the response to ARV therapy and development of drug resistance (Chapters 3 and 4). Additionally, we looked for the mutations associated with drug resistance and estimated the rate of TDR in these populations.

In Cape Verde we worked with treated and untreated patients. Thus, besides investigating TDR we also investigated acquired drug resistance and explored the relationship to each other (Chapter 5). Up to this day, no studies have been published on HIV-1 diversity in Cape Verde; hence, we attempted to initially fill this gap by genotyping the isolates of a small number of HIV-1 infected patients living in Santiago Island (Chapter 5). Finally, we present the first genetic characterization of HIV-2 isolates circulating in this Island.

Likely due to close ties with Angola, Mozambique and Cape Verde, the HIV-1/AIDS epidemic in Portugal is caused by multiple subtypes with predominance of subtype B (41.7%) followed by

subtype G (29.4%). The first recombinant between these two subtypes (CRF14_BG) was detected in Portugal in 1998 in an individual that did not seroconvert and died of AIDS in only seven months after infection. In Chapter 6 the investigation of this case of seronegative infection is described and an initial characterization of this BG virus is presented based on partial clonal sequences from the *gag* and *env* genes. To fully characterize CRF14_BGs from Portugal and reconstruct the evolutionary history of this CRF, three near full-length genomic sequences were obtained and molecular clock analysis was performed (Chapter 7). Finally, to gain some insight into the biological and genetic determinants of the highly aggressive phenotype of most isolates belonging to this CRF, genetic and pyhlogenetic methods were used to determine the tropism of a significant number of CRF14_BG isolates from Portuguese HIV-1 infected patients, and to investigate positive selection in the V3 region (Chapter 7).

CHAPTER 2

Highly divergent subtypes and new recombinant forms prevail in the HIV/AIDS epidemic in Angola: new insights into the origins of the AIDS pandemic

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Abstract

Angola, located in South-Western Africa, has a remarkably low HIV/AIDS prevalence in the adult population (3.7%). It is bordered in the North by the Democratic Republic of Congo (DRC) and Republic of Congo that are at the origin of human HIV-1 infections. It is, therefore, likely that HIV-1 strains circulating in Angola are genetically diverse and representative of the origin of the HIV/AIDS epidemic. The aim of this work was to investigate in more detail the genetic diversity and molecular epidemiology of HIV-1 in Angola. Almost 400 sequences were obtained from the *gag* (p17), *pol* (PR and RT) and/or *env* (C2C3) genes of 159 HIV-1 infected patients living in eight provinces of Angola (Benguela, Cabinda, Cuanza Norte, Luanda, Lunda Norte, Malange, Uíge, and Zaire) and their genotype was determined by phylogenetic analyses. Gene regions representing all HIV-1 group M clades were found as well as unclassifiable sequences. In *env* and *pol* (RT), two groups of sequences forming distinct sub-clusters within the subtype A radiation were found and may define new A5 and A6 sub-subtypes. Recombinant forms were found in almost half (47.1%) of the patients of which 36.0% were second-generation recombinants. Fifty eight different patterns of recombination were found. The A subtype, including CRF02_AG, was represented in most recombinant viruses. Epidemiological data suggests that the AIDS epidemic in Angola has probably started as early as 1961, the major cause being the independence war, and spread to Portugal soon thereafter. The extraordinary degree of HIV-1 group M genetic diversity and evolution in Angola may pose unprecedented challenges to diagnostic, treatment and prevention of HIV-1 infection.

Introduction

More than 22.5 million people are living with HIV/AIDS in Sub-Saharan Africa, and this accounts for the fastest moving AIDS epidemic worldwide. In 2007, an estimated 1.7 million people in the region became newly infected, while 1.6 million adults and children died of AIDS [533].

Angola is a South-western African country bordered by Republic of Congo, Democratic Republic of Congo (DRC), Zambia and Namibia. According to the UNAIDS epidemiological fact sheets, 2006 update, the estimated number of adults and children living with HIV/AIDS in Angola was 320,000, and the number of deaths due to AIDS 30,000 [534]. The HIV/AIDS prevalence was 3.7% in the adult population which is very low as compared to Southern Africa countries such as Republic of South Africa (18.8%, 2005 estimate) [535] yet similar to the neighbouring country DRC (3.2%) [536]. HIV-1 prevalence in Angola has been decreasing since 1989 as judged by the 6.1% prevalence detected at that time in a major nationwide seroprevalence study [537].

HIV-1 genetic forms have been divided into three main groups, M (major), O (outlier) and N (new). Among HIV-1 group M strains, responsible for the vast majority of HIV infections worldwide, there are 9 genetic subtypes (A-D, F-H, J and K), 6 sub-subtypes (A1, A2, A3 and A4 and F1 and F2), 37 circulating recombinant forms (CRFs) and a variety of unique recombinant forms (URFs) [52, 60, 538]. Second-generation recombinants combining one or more CRFs with different subtypes were first identified in patients from Cameroon [202] and are becoming common in complex epidemics with multiple subtypes and recombinant forms such as Cuba [89] and Cameroon [60, 61].

HIV-1 group M arose from SIVcpz infecting the chimpanzee subspecies *Pan troglodytes troglodytes* in Cameroon, West Central Africa [31]. The common ancestor of HIV-1 group M in humans dates back to the 1940s and the beginning of the HIV-1 group M epidemic is estimated to be the 1960s [17]. However, the factors that have spurred worlwide HIV-1 transmission are still unknown.

The global distribution of HIV is complex and dynamic with regional epidemics representing only a subset of the global diversity [105, 539]. HIV genetic forms differ significantly in terms of global prevalence. As expected, the highest genetic diversity is observed in West Central Africa; for example, all HIV-1 groups co-circulate in Cameroon [540-542]. The AIDS epidemic in Western DRC is caused by all HIV-1 group M genetic forms (subtypes and recombinants). This epidemic is also characterized by high intra-subtype diversity, high proportion of unique recombinant forms, and a substantial number of genetic forms that cannot be classified into the current subtypes [50, 159, 543]. High degree of HIV-1 genetic diversity is also found in countries with former ties with Central Africa. For instance, the Cuban [89, 196, 544] and Portuguese [160, 162, 187] AIDS epidemics are characterized by high proportion of all non-B subtypes, CRFs and multiple recombinant forms between these variants.

The rapid evolution and redistribution of HIV pose a significant challenge to the reliability of screening, diagnostic, and patient monitoring assays [105, 312]. The ability to anticipate and respond to this threat will be dictated to a significant degree by our level of vigilance for newly emerging strains. The diversity of HIV-1 genetic forms also challenges the prevention and treatment

programs worldwide. The differences among HIV-1 genetic forms may have a profound impact on clinical management and surveillance of drug resistance, particularly as treatment is expanded to non-subtype B viruses [105, 545]. For instance, CRF02_AG may be more susceptible to nelfinavir and ritonavir than other subtypes and this was associated with the 70R polymorphism. Susceptibility to tipranavir may be lower among the subtype F and higher for subtype G [546].

Previous studies with a limited number of patients and restricted geographical and gene coverage indicated that most HIV-1 subtypes are present in Angola [167, 198]. The aim of this work was to perform a detailed follow up investigation of the genetic diversity and molecular epidemiology of HIV-1 in Angola.

Material and Methods

Patients

Samples were collected from subjects with positive HIV serology in 2001, except for seven samples that were collected in 1993. Sequences were obtained from *gag* (p17), *pol* (PR and RT) and/or *env* (C2C3) genes of 159 HIV-1 infected patients living in the following provinces of Angola: Benguela (1 patient, 0.6 %), Cabinda (22, 13.8 %), Cuanza Norte (1, 0.6 %), Luanda (126, 79.3 %), Lunda Norte (3, 1.9 %), Malange (2, 1.3 %), Uíge (1, 0.6 %), and Zaire (3, 1.9 %). The serological diagnostic of the patients was done in Angola and the results were confirmed in our lab with GENSCREEN® PLUS HIV Ag-Ab (BIO-RAD). The epidemiological characterization of the patients is described in Table 2.1. According to the CDC Classification System for HIV Infection category A consists in asymptomatic HIV infection or acute (primary) HIV infection; category B consists of symptomatic conditions that are not included among conditions listed in clinical category C and that meet at least one of the following criteria: the conditions are attributed to HIV infection or are indicative of a defect in cell-mediated immunity or the conditions are considered by physicians to have a clinical course or to require management that is complicated by HIV infection; and category C includes the clinical conditions listed in the AIDS surveillance case definition [547].

Viral RNA Extraction, PCR Amplification and Sequencing

Sequences were obtained from *gag* (p17), *pol* (PR and RT) and/or *env* (C2C3) genes of 159 HIV-1 infected patients. RNA was extracted from 200 μl of plasma using Nuclisens Isolation Kit (BioMerieux). RT-PCR was performed with Titan One Tube RT-PCR System (Roche). Nested PCR was done to obtain a 409 bp fragment from the C2C3 *env* region using outer primers JA167 and JA170 and inner primers JA168 and JA169; to obtain a 582 bp fragment from the p17 *gag* region we used outer primers JA152 and JA155 and inner primers JA153 and JA154. Thermal cycling conditions for amplification of C2C3 *env* region and p17 *gag* region and primer numbers and positions have been described previously [548]. Nested PCR was done to obtain a 532 bp fragment from the PR region using outer primers IBPR1.1 and IBPR2.2 and inner primers IBPR3.1 and IB2621PR4; to obtain a 1026

bp fragment from the RT region we used outer primers IB2480RT1 and IB3626RT2 and inner primers IB2530RT3 and IB3555RT4. PCR and primer numbers and positions are described in Table 2.2. DNA sequences were obtained with Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems) using the primers described in Table 2.2.

TABLE 2.1

Epidemiological and Clinical Characteristics of the Angolan Samples

Parameters	Number of patients	Percentage
Age group (years)		
Median (range)	40 (7-69)	
≤ 14	4	2.5
15-49	112	70.4
≥50	23	14.5
Unknown	20	12.6
Gender		
Male	62	39.9
Female	92	57.9
Unknown	5	3.1
Transmission route		
Sexual	115	72.3
Heterosexual	109	68.6
MSM[a]	1	0.6
Bisexual	5	3.1
Vertical	5	3.1
Parenteral	4	2.6
Blood transfusion	2	1.3
IDU[b]	2	1.3
Unknown	35	22.0
CDC clinical stage		
A	30	18.9
B	55	34.6
C	31	19.5
Unknown	43	27.0

[a] Men who have Sex with Men; [b] Intravenous Drug User.

Phylogenetic and Recombination Analysis

Sequences were aligned with reference strains [538] from each subtype using ClustalX 1.8 [195]. All sequences from neighbouring countries available in the databases, especially those from DRC and Republic of Congo, were included in the alignment. The genetic distances between sequences were calculated using the Kimura two parameter substitution model with pairwise gap deletion [549] as implemented in the MEGA 3.1 program [550]. Maximum likelihood phylogenetic analyses [551] were performed using the best-fit model of molecular evolution estimated by Modeltest v3.7 under the Akaike information criterion [552]. The chosen models were K81uf+I+G for the *env* gene and

GTR+I+G for *gag* and *pol* genes. Tree searches were conducted in PAUP v4.0b10 using a nearest-neighbor interchange (NNI) heuristic search strategy [553], and bootstrap resampling [554].

TABLE 2.2

PCR and Sequencing Primers Used for the *pol* Gene

Name	Position[a]	Region	Sequence (5´- 3´)
IBPR1.1[b]	2008-2030	PR	AAAAGGGCTGTTGGAAATGTGG
IBPR2.2 [b]	2733-2712	PR	GCAAATACTGGAGT(A/G)TT(G/A)TATG
IBPR3.1[c,d]	2119-2140	PR	AGGCCAGGGAATTT(T/C)C(T/C)TCAGA
IB2621PR4 [c,d]	2650-2621	PR	AATGCTTTTATTTT(C/T)TCTTCTGTCAATGGC
IB2480RT1[b]	2480-2499	RT	AGTAGGACCTACACCTGTC
IB3626RT2[b]	3593-3626	RT	TCCGTTAA(C/T)TGT(C/T)TTACATCATTAGTGTG(A/G)GCA
IB2530RT3[c]	2530-2565	RT	TTGGTTG(C/T)ACTTTAA ATTTTCCAATTAGTCC(C/T)ATT
IB3555RT4[c]	3526-3555	RT	GG(C/T)TCTTG(A/G)TAAATTTGATATGTCCATTG
IB2604RT5[d]	2604-2633	RT	CCAAA(A/G)GTTAAACAATGGCCATTGACAGA
IB3555RT6[d]	3555-3538	RT	ATTTGATATGTCCATTG
IB2997RT7[d]	2997-3020	RT	CCACAGGGATGGAAAGGATCACC
IB2997RT8[d]	2997-2974	RT	GGTGATCCTTTCCATCCCTGTGG

[a] HIV1 - HXB2; [b] Outer primer for PCR; [c] Inner primer for PCR; [d] Sequencing primer.

Bootstrap values ≥70% were considered definitive for significant clustering [555]. To confirm recombination events and identify recombination breakpoints, bootscanning analysis was performed using Simplot 3.5.1 [556]. Briefly, SimPlot calculates and plots the percent identity of the query sequence to a panel of reference sequences from group M viruses in a sliding window, which is moved across the alignment with optimal step size. The window and step sizes are adjustable [557]. The simplot software performed bootscanning on neighbor joining trees by usind SEQBOOT, DNADIST (with Kimura´s two-parameter method and F84 maximum likelihood model, transition/transversion ratio = 2), NEIGHBOR, and CONSENSE from the PHYLIP package for a 200 bp window moved along the aligment in increments of 20 bp.

Statistical Analysis

Statistical analysis was performed in GraphPad Prism version 4.0 [558] with a level of significance of 5%. Inter and intra-subtype genetic distances were compared using the unpaired Student t test.

GenBank Accession Numbers

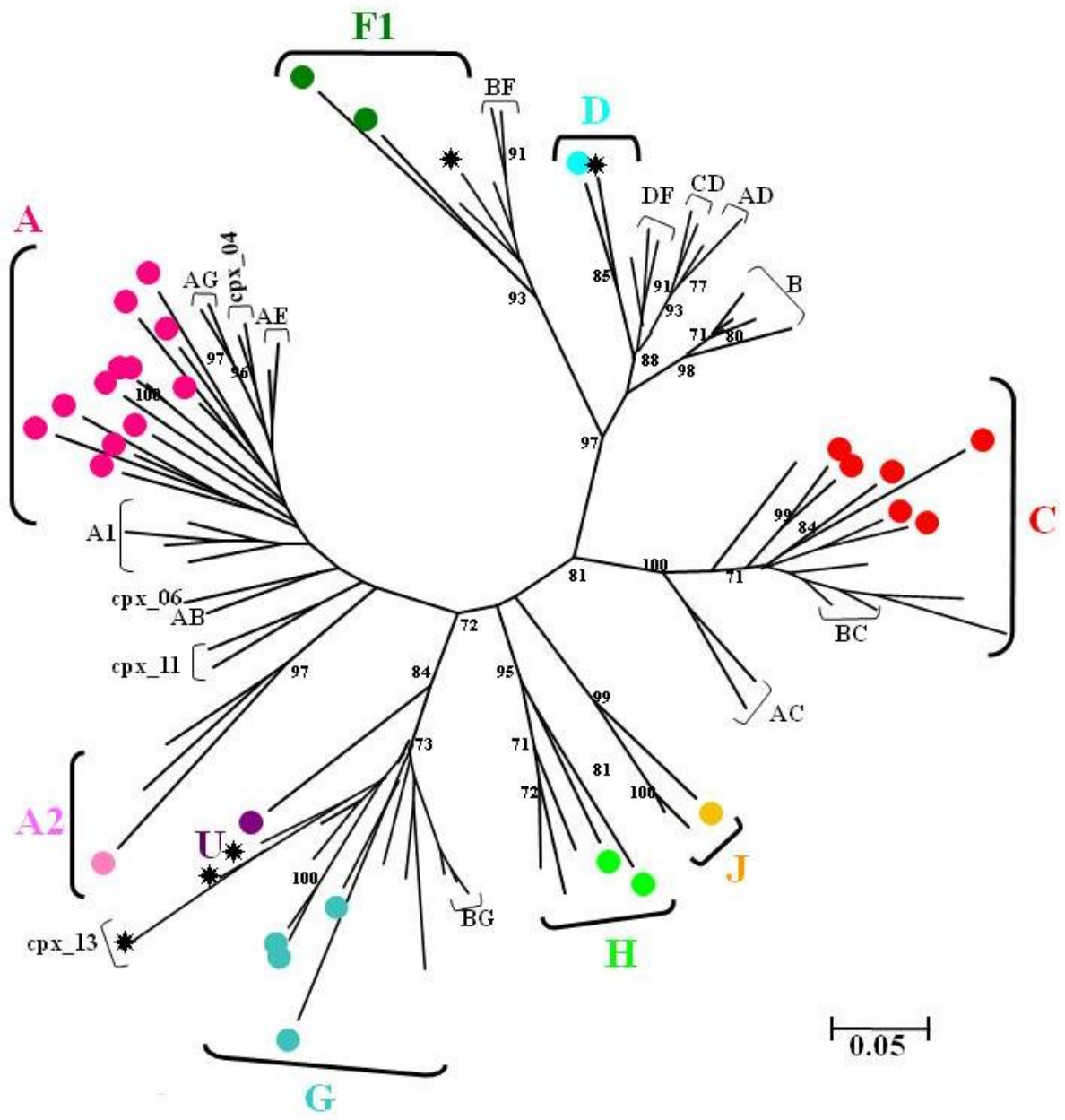
Sequences have been assigned the following GenBank accession numbers AY456278-AY456330, AY676573-AY67594, EU031839-EU031891 and EU068199-EU068462.

Results

The majority of the patients analysed in this study were from Luanda (126, 79.3 %), the Capital city, followed by Cabinda (22, 13.8 %). A few patients were from seven other Provinces located mostly in the northern part of the country, Benguela (1, 0.6 %), Cuanza Norte (1, 0.6 %), Lunda Norte (3, 1.9 %), Malange (2, 1.3 %), Uíge (1, 0.6 %), and Zaire (3, 1.9 %) (Figure 2.3). The median age of the patients was 40 years (7-69 years) and 57.9% were women (Table 2.1). Heterosexual contact was the most common transmission route (68.6%). According to the CDC Classification System for HIV Infection [547], only 18.9% of the patients were in an asymptomatic condition, CDC clinical stage A, 34.6% were in clinical stage B and 19.5% were in clinical stage C (AIDS), at the time of sampling. These results are consistent with the fact that none of these patients were taking antiretroviral drugs.

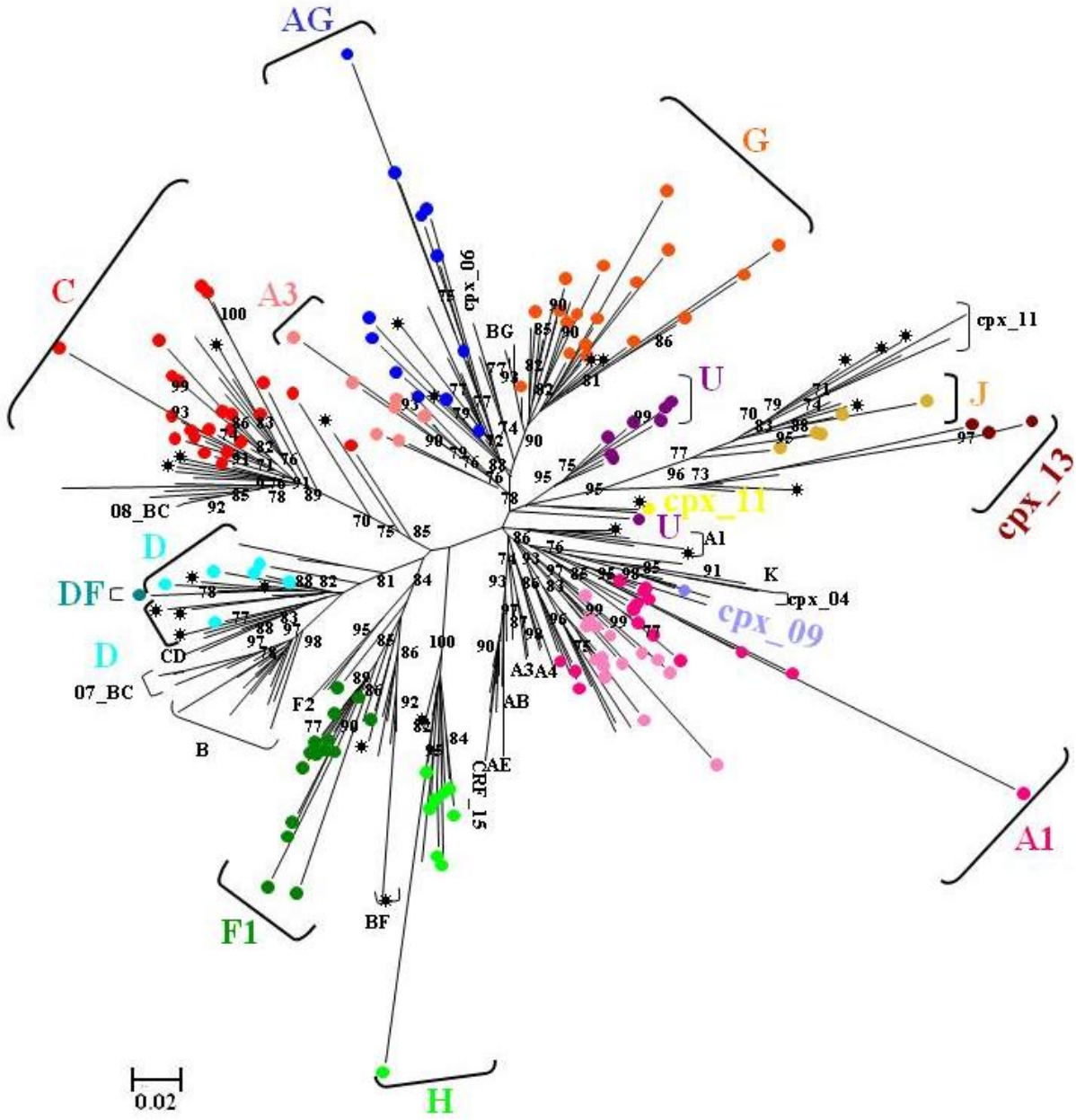
A total of 390 sequences were produced from p17 (30), PR (141), RT (123) and C2C3 (96) and phylogenetic analysis was used to genotype the sequences (Figure 2.1). Table 2.3 summarizes the genetic forms found in each patient and each gene region.

No group N or O viruses were found. Gene regions representing all HIV-1 group M clades were found, as well as unclassifiable (7.5%) sequences. The following pure subtypes and sub-subtypes were identified: A1 (8.8%), A2 (4.4%), A3 (0.6%), C (11.3%), D (5.0%), F1 (8.2%), G (5.7%), H (5.7%) and J (3.1%). Two or more genomic regions were sequenced in 122 patients. Discordant phylogenies among the different genes indicating recombination were found in 75 (47.1%) patients (Table 2.3). Fifty eight different patterns of recombination were found. There were 27 (36.0%) second generation recombinant viruses, of which the most prevalent was A3/CRF02_AG/A1 (PR/RT/C2C3) (3, 11.1%). A1, A2 and A3 sub-subtypes and CRF02_AG were involved in most of the recombinant events (53.3%). Bootscaning analysis of concatenated genes confirmed the recombination events and enabled the identification of some recombination breakpoints. Figure 2.2 presents three examples of the results obtained with the bootscaning analysis. The isolate from patient 01AOSNS03 is a recombinant between subtype C (in p17) and sub-subtype A2 (in PR, RT and C2C3) (Figure 2.2 A). In the isolates 01AOSNS09 and 01AOHDP29 besides the recombination between genetic regions, we found evidence of recombination within regions. Thus, in the RT region 01AOSNS09 (Figure 2.2 B) is a U/J/U recombinant and has recombination breakpoints in the 1340 and 1550 bp positions; 01AOHDP29 (Figure 2.2 C) is a recombinant A3/U in the PR region, with the recombination breakpoint in the position 200 bp. The genetic classification of each region was confirmed by phylogenetic analysis performed with a sliding window around the recombination breakpoints (data not shown).



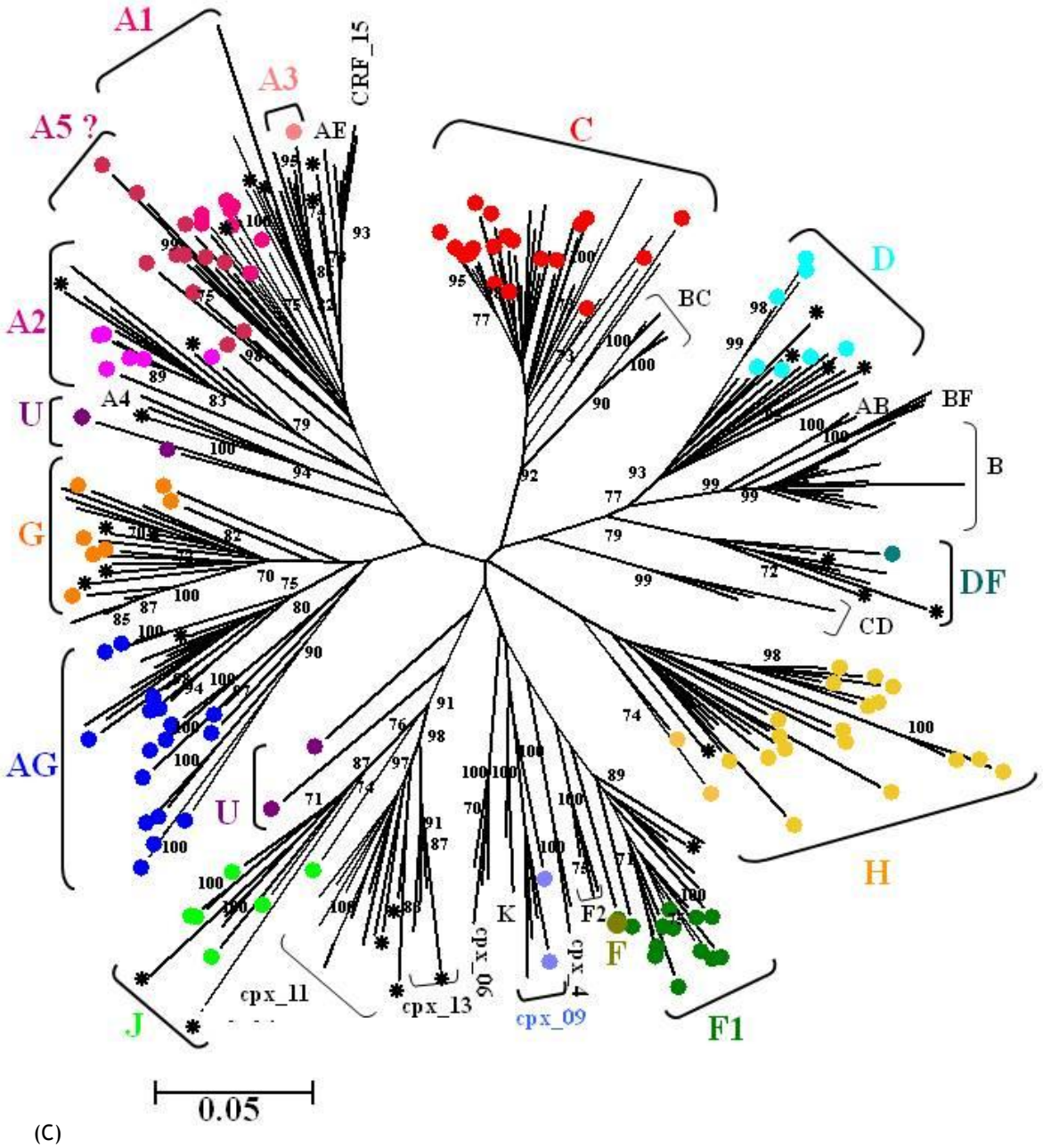
(A)

FIGURE 2.1 - Genetic subtypes and evolutionary relationships of the viruses sequenced in this study based on maximum likelihood phylogenetic trees of partial *gag* (p17) (A), *pol* PR (B), *pol* RT (C) and partial *env* sequences (C2C3) (D). The phylogenetic trees were constructed under a Kimura three-parameters with unequal base frequencies (K81uf +I+G) model of evolution for the *env* gene and under GTR+I+G for *gag* and *pol* genes. The phylogenetic trees were constructed with reference sequences from all HIV-1 subtypes and sub-subtypes as well as with the Angolan sequences (shown in coloured symbols) and DRC sequences (indicated with asterisks). Putative new A5 and A6 sub-subtypes are indicated with interrogation marks. The bootstrap values supporting each of the internal branches defining a subtype or a sub-subtype are shown. The scale represents number of base substitutions per site.



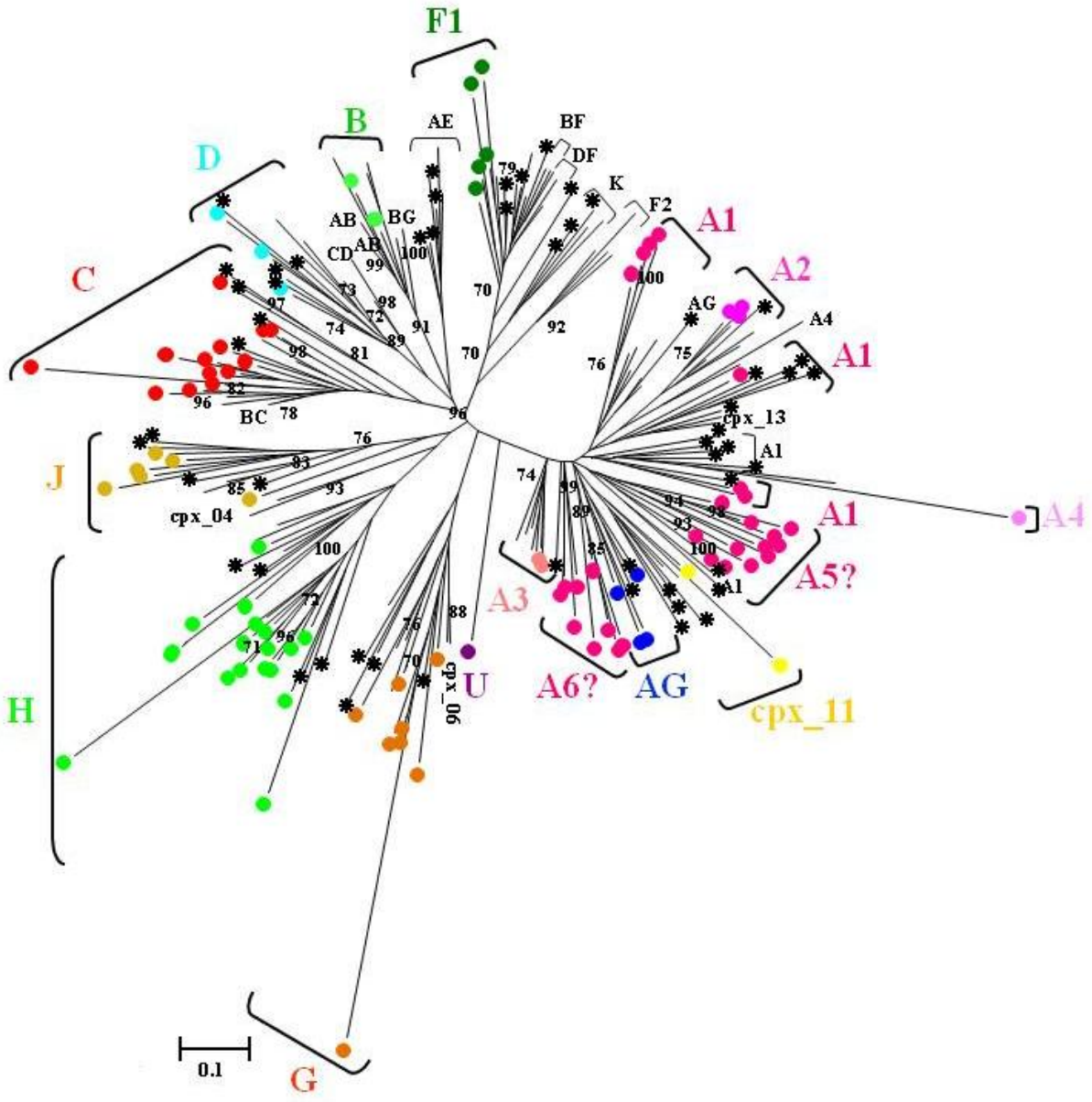
(B)

FIGURE 2.1 *(continued)*



(C)

FIGURE 2.1 *(continued)*



(D)

FIGURE 2.1 *(continued)*

TABLE 2.3

HIV-1 Genetic Forms Found in this Study in Angolan Patients

Isolate	Genotype				Isolate	Genotype			
	p17	PR	RT	C2C3		p17	PR	RT	C2C3
01AOSNS01	U[a]	G	H	H	01AOHJM62	-	09cpx	09cpx	11cpx
01AOSNS02	-	G	-	-	01AOHJM63	-	G	02AG	H
01AOSNS03	C	A_2	A_2	A_2	01AOHJM64	C	C	C	C
01AOSNS04	-	A_2	A_1	-	01AOHDP65	-	02AG	-	-
01AOHJM06	A_1	A_1	H	H	01AOHDP66	-	02AG	02AG	02AG
01AOSNS09	C	G	U[a]	C	01AOHDP67	A_1	02AG	G	A_1
01AOSNS10	-	-	05DF	-	01AOHDP68	-	C	C	A_1
01AOLFA11	A_1	G	02AG	A_1	01AOHDP69	-	C	-	-
01AOLFA12	-	-	-	A_1	01AOHDP70	-	C	H	H
01AOLFA13	-	F_1	F_1	-	01AOHDP71	-	C	C	C
01AOLFA14	A_2	A_2	A_2	A_2	01AOHDP72	-	A_1	A_1	J
01AOLFA15	C	C	C	C	01AOHDP73	-	A_1	H	02AG
01AOLFA16	-	C	C	C	01AOHDP74	-	A_1	-	-
01AOLFA17	A_1	A_1	A_1	A_1	01AOHDP75	A_1	02AG	02AG	A_3
01AOLFA18	-	A_2	A_2	-	01AOHDP76	-	U[a]	-	-
01AOLFA19	A_1	A_1	A_1	A_1	01AOHDP78	-	A_1	-	-
01AOHSL20	-	F_1	F_1	-	01AOHAB79	-	G	H	H
01AOSNS21	-	C	C	C	01AOHAB80	-	C	H	C
01AOSNS22	A_1	U[a]	H	H	01AOHAB81	-	A_2	-	A_1
01AOSNS23	A_1	G	C	H	01AOHAB82	-	02AG	-	A
01AOSNS24	-	A_3	A	A	01AOHAB83	-	F_1	C	C
01AOSNS25	-	A_1	A_1	A_1	01AOHAB84	-	-	-	A
01AOSNS27	-	-	02AG	A_1	01AOHAB85	-	F_1	C	F_1
01AOSNS28	-	-	D	-	01AOHAB86	-	F_1	F_1	-
01AOHDP29	-	U[a]	H	H	01AOHAB87	-	A_2	-	-
01AOHJM31	-	A_2	-	-	01AOHAB89	G	13cpx	-	B
01AOSNS32	-	U[a]	09cpx	A_4	01AOHAB90	-	A_3	02AG	A_1
01AOSNS33	-	-	D	-	01AOHAB91	G	G	G	G
01AOSNS35	-	A_1	-	A_1	01AOHAB92	G	13cpx	-	J
01AOSNS36	-	A_2	A	A	01AOHAB93	-	A_2	-	-
01AOSNS37	-	G	G	-	01AOLFA94	-	A_2	A	A
01AOHSL38	-	A_1	A	-	01AOLFA95	-	U[a]	02AG	H
01AOSNS39	-	A_1	A_1	A_1	01AOLFA96	-	A_1	J	A_1
01AOSNS40	A_1	02AG	02AG	A_1	01AOLFA97	-	02AG	02AG	A_1
01AOSNS41	.	-	F_1	-	01AOLFA98	-	A_1	-	U[a]
01AOSNS47	.	G	G	G	01AOLFA99	-	U[a]	-	-
01AOSNS48	.	F_1	F_1	F_1	01AOHDP100	-	G	G	G
01AOSNS49	.	A_3	02AG	A_1	01AOHJM101	-	02AG	-	G
01AOSNS50	.	J	J	-	01AOHSN102	-	-	02AG	-
01AOSNS51	.	H	H	H	01AOSNS104	-	F_1	-	-
01AOSNS52	-	G	G	H	01AOSNS106	-	A_3	A_1	A_1
01AOSNS53	-	A_1	-	-	01AOSNS107	-	02AG	U	A_1
01AOSNS54	-	G	-	-	01AOSNS108	-	-	02AG	-
01AOSNS55	C	C	C	C	01AOSNS110	-	G	-	G
01AOSNS56	-	H	H	H	01AOHDP111	-	-	-	G
01AOSNS59	C	C	C	C	01AOHDP112	-	A_1	A	H
01AOHJM60	-	C	-	-	01AOHJM113	-	D	-	-
01AOHJM61	-	13cpx	U[a]	A_2	01AOHJM114	-	D	-	J

TABLE 2.3 *(continued)*

Isolate	Genotype				Isolate	Genotype			
	p17	PR	RT	C2C3		p17	PR	RT	C2C3
01AOHJM115	-	C	C	C	01AOHM189	-	U[a]	H	-
01AOCSE116	-	F_1	C	F_1	01AOHM191	-	A_2	A_2	-
01AOCSE117	-	A_1	A_1	A_1	01AOHM192	-	C	C	-
01AOCSE118	-	A_3	02AG	A_1	01AOHM193	-	A_2	A_2	-
01AOCSE120	-	02AG	U	H	01AOHM194	-	C	C	-
01AOCSE121	-	D	D	D	01AOHM195	-	02AG	A_2	-
01AOCSE122	-	C	C	C	01AOHM198	-	H	H	-
01AOCSE123	-	C	C	C	01AOHM199	-	D	D	-
01AOCSE124	-	A_2	A	A	01AOHM200	-	F_1	F_1	-
01AOCSE125	-	02AG	02AG	A_3	01AOHDC228	F_1	F_1	F_1	B
01AOCSE126	-	F_1	F_1	F_1	01AOHDC229	A_1	G	H	G
01AOCSE127	-	U[a]	H	H	01AOHDC230	H	H	H	H
01AOCSE128	-	F_1	-	-	01AOHDC231	A_1	-	-	-
01AOCSE129	-	C	-	-	01AOHDC232	G	G	G	H
01AOCSE130	-	J	-	-	01AOHDC233	-	H	H	-
01AOCSE133	-	-	G	-	01AOHDC234	H	H	-	-
01AOCSE134	-	C	G	11cpx	01AOHDC235	A_1	A_1	02AG	A_1
01AOCSE135	-	-	A_1	-	01AOHDC236	J	J	J	J
01AOCSE136	-	A_1	A_3	02AG	01AOHDC237	-	H	H	H
01AOCSE140	-	-	-	A_1	01AOHDC238	-	D	D	-
01AOCSE141	-	-	-	G	01AOHDC239	D	05DF	D	D
01AOML143	-	-	F_1	-	01AOHDC240	F_1	F_1	F_1	F_1
01AOINSP145	-	-	-	D	01AOHDC241	-	A_1	J	J
01AOLFA146	-	-	F_1	-	01AOHDC242	-	G	A	02AG
01AOHM176	-	D	D	-	93AOHDC247	-	J	J	J
01AOHM180	-	C	C	-	93AOHDC248	-	H	H	-
01AOHM182	-	C	C	-	93AOHDC249	-	A_3	A	-
01AOHM183	-	C	C	-	93AOHDC250	-	A_3	-	-
01AOHM184	-	G	H	-	93AOHDC251	-	H	H	-
01AOHM185	-	A_1	A_1	-	93AOHDC252	-	A_3	A	-
01AOHM186	-	F_1	F_1	-	93AOHDC253	-	J	J	-
01AOHM187	-	F_1	F_1	-					

[a] Untypable; (-) - Unknown;

In the *env* and RT, two groups of sequences forming distinct sub-clusters within the subtype A radiation were found (Figure 2.1 C and D). Genetic distances to sub-subtype A1, A2 and A3 varied from 19.3 to 25.2% and 16.4 to 22.7% for the two sub-clusters in the *env* gene and from 8.3 to 10.6% for the sub-cluster in the RT region. The high genetic distances suggest that these sequences may define new A5 and A6 sub-subtypes.

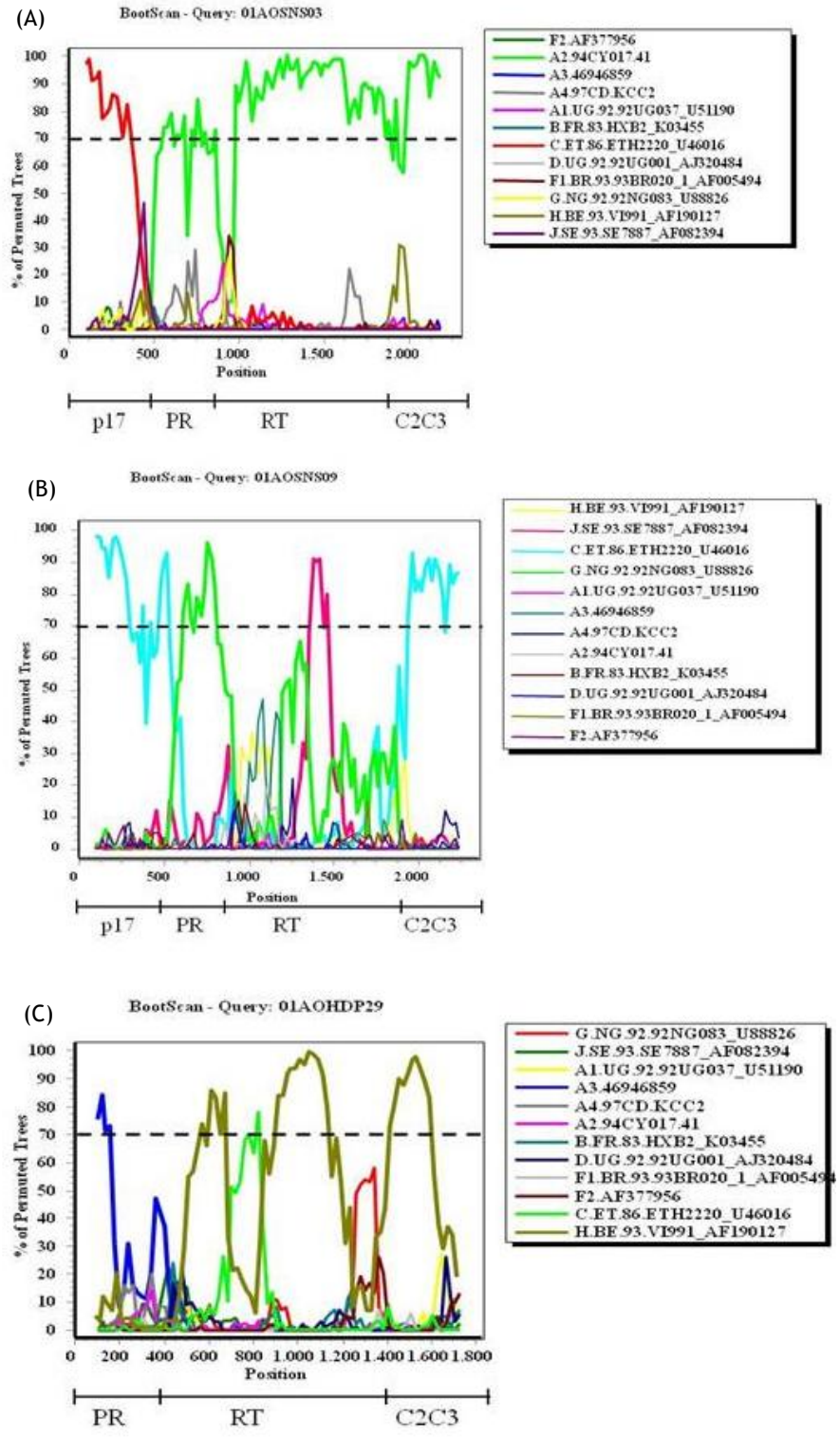


FIGURE 2.2 - Sliding window bootscanning analysis (window 200bp; step 20bp) of Angolan isolates 01AOSNS03 (A), 01AOSNS09 (B) and 01AOHDP29 (C). Bootscanning analysis was done as described in the text. The dashed line indicates the cut-off of 70%.

Discussion

This is the first work to document the genetic diversity of HIV-1 in a large number of patients living in multiple Angolan Provinces. In a significant amount of seropositive samples (52) we could not amplify viral DNA. This is not uncommon in this region of Africa and is probably related to the tremendous genetic variability of HIV-1 [52, 159, 312]. A total of 159 samples were directly sequenced in *gag* (p17), *pol* (PR and RT) and *env* (C2C3) genes. The phylogenetic analyses showed extremely high genetic diversity among Angolan HIV-1 strains. In Angola all HIV-1 group M subtypes and sub-subtypes co-circulate as well as the following CRFs: 02_AG, 05_DF, 06_cpx, 09_cpx, 11_cpx and 13_cpx. Only 53% of viruses were pure subtypes, subtype A and its sub-subtypes predominating over the other subtypes (13.8%). If we consider that CRF02_AG is indeed an A sub-subtype and G is a recombinant between the A and J subtypes [559] then these figures will be even higher (19.5% adding subtype G and 22.0% adding CRF02_AG). This was not surprising because the countries in the Northern border of Angola have a predominance of the A subtype: 46-48% in DRC [159, 543] and 37-48% in Republic of Congo [560, 561] (Figure 2.3). In Zambia, subtype C prevails over the other subtypes (98%) [562] so the significant proportion of subtype C that was found in our patients (11.3%) was also expected. Finally, of note was the detection of the rare H (5.7%) and J (3.1%) subtypes and almost eight percent of unclassifiable sequences.

In the *env* and *pol (*RT) genes, two groups of sequences forming distinct sub-clusters within the subtype A radiation were found (Figure 2.1). The values that we found for the genetic distances to sub-subtype A1, A2 and A3 sequences for the two sub-clusters in the *env* gene and for the sub-cluster in the RT region are similar to the ones observed within the other sub-subtypes for the same regions [48]. These sequences may, therefore, define new A5 and A6 sub-subtypes.

Like in DRC, we found that a number of our sequences fall at basal positions on the phylogenetic trees (pre-subtype branches) [159, 563, 564]. In addition, some strains from Angola have little organized substructure and form weaker clusters within phylogenetic trees than the global reference sequences, not allowing a clear distinction between subtypes. As a consequence, the current global subtype classification may not reflect the extent of diversity in this region [35]. Further analyses with genomic sequences will be needed to clarify whether these Angolan strains are ancestor viruses, pure subtypes or represent new genetic forms of HIV-1. Overall, these results indicate that Angola, like DRC, is one of the epicenters of the epidemic, with the global subtypes resulting from the export of some Angolan strains to other geographical regions.

Viral recombination takes place in geographic regions where several subtypes and CRFs circulate in the population. In these regions, 8-24% of the infections may be due to inter-subtype recombinants [48, 565]. In this study the percentage of recombinant strains was almost twice that amount (47.1%). Moreover, the Angolan recombinants are extremely complex since 58 different patterns of recombination were identified and 36.0% of these were second-generation recombinants. Second-generation recombinants were described for the first time in patients from Cameroon [202]. Recent studies performed in this country have led to the discovery of two new CRFs, CRF36_cpx and

CRF37_cpx, which combine two CRFs, CRF01_AE and CRF02_AG [60, 61]. High number of recombinant strains imply high rate of co-infections and super-infections which is surprising given the current low prevalence of HIV-1 in Angola. It may be that the current HIV/AIDS epidemic in Angola is mostly driven by a subpopulation of highly exposed individuals (such as sex workers) that are more prone to superinfection and give rise to higher indidences of recombination than could be expected from overall prevalence only.

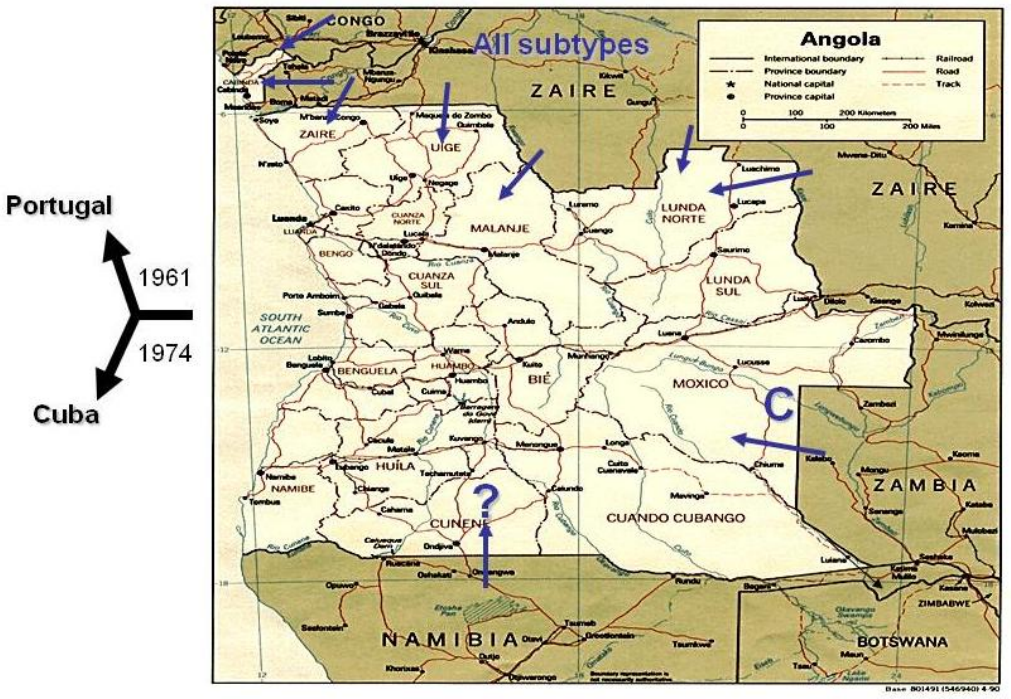


FIGURE 2.3 - Possible origins of the Angolan HIV-1 genetic forms and estimated dates of their dissemination to Portugal and Cuba.

On the other hand, our results would be easy to explain if recombinant strains were the ancestors of the current subtypes as it has been recently demonstrated for CRF02_AG and subtype G [559].
There is a direct association between HIV-1 genetic diversity, origin and infection time, so that older epidemics with multiple focus of infection, like HIV-1 infection in Africa, are characterized by viruses with high genetic diversity [50, 159, 541, 543, 561, 566], while recent infections originated by a single viral population are characterized by viruses with low among-host genetic diversity [112, 179, 456, 567, 568]. In this context, the high genetic diversity of HIV-1 found in Angola suggests that, on one hand, HIV-1 is circulating in Angola for a long time and, on the other hand, that there is intense population mobility between Angola, DRC and Republic of Congo [537]. Two major events may have contributed significantly to the pronounced genetic complexity of HIV-1 in Angola: the colonial war with the Portuguese and the civil war following Portuguese decolonisation. During the first years of the colonial war, starting in March 1961 and ending in April 1974, thousands of people

temporarily fled from Angola to the neighbouring countries, especially to DRC and Republic of Congo. HIV-1 group M has been circulating in DRC probably since the 1940s [17, 41]. It is, therefore, likely that an important number of Angolan subjects become infected with HIV-1 in 1961 in the South of DRC and Republic of Congo bringing back into Angola the different genetic forms of HIV-1 present in those countries at the end of 1961 beginning of 1962, when the population returned to Angola (Figure 2.3). This implies that the HIV-1 epidemic in Angola is coincident with the beginning of the HIV-1/AIDS group M epidemic, which is estimated to be the 1960s [17]. Importantly, the independence war in Angola may have been the exact extrinsic factor that started the HIV-1/AIDS epidemic worldwide, much like the independence war in Guinea-Bissau (1963-1974) was the extrinsic factor that started the HIV-2 epidemic [17]. The epidemiologic data and the very high intra-subtype divergence of Angolan HIV-1 isolates are consistent with a long standing HIV-1 epidemic in this country [537]. On the other hand, the finding that the AIDS epidemic in Portugal is caused by a high proportion of highly divergent non-B subtypes and recombinant forms, strongly suggests that non-subtype B strains have been present in Portugal for a long time [160, 162, 187]. The combined data, therefore, suggest that the intense people displacements during the colonial war with Angola may have had a decisive contribution to start the AIDS epidemic in Angola and Portugal, possibly in the mid-1960s. The civil war following Portuguese decolonisation, starting in April 1974 and ending 2002, again prompted delocalization of large number of people within Angola and from Angola to the neighbouring countries specially DRC and Republic of Congo [537, 569]. This may also have helped to spread HIV-1 variants from DRC and Republic of Congo into Angola (Figure 2.3). As it has happened in Portugal during the independence war, the presence of Cuban soldiers in Angola in the civil war probably contributed significantly to shape the current AIDS epidemic in Cuba, which is in part caused by highly divergent non-B HIV-1 isolates and recombinant forms of African origin [196, 544, 569].

In conclusion, the extraordinary degree of HIV-1 genetic diversity in Angola is probably related to its close geographical proximity with several countries in Central Africa with old human HIV-1 infections such as the DRC and Republic of Congo. The HIV/AIDS epidemic in Angola probably started in 1961, the major cause being the independence war, and subsequently spread to Portugal. The fast rate of HIV-1 group M genetic evolution detected in Angola may pose unprecedented challenges to diagnostic, treatment and prevention of HIV-1 infection.

Acknowledgments

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CHAPTER 3

Antiretroviral drug resistance surveillance among treatment-naive HIV-1-infected individuals in Angola: evidence for low level of transmitted drug resistance

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Abstract

The prevalence of transmitted HIV-1 drug resistance in Angola in 2001 was investigated in 196 untreated patients. All subtypes were detected, along with unclassifiable and complex recombinant strains. Numerous new polymorphisms were identified in the RT and PR. Two (1.6%) unrelated patients harboured NRTI and NNRTI resistant viruses (mutations: M41L, D67N, M184V, L210W, T215Y or T215F and K103N). Continued surveillance of drug resistance is required to maximize ART efficacy in Angola.

The AIDS epidemic in Angola, a South-Western African country, is caused by HIV-1 strains exhibiting high genetic diversity [167, 570]. By the end of 2007, the HIV/AIDS prevalence in the adult population was 2.1% (1.7%-2.5%) [571].
Undocumented antiretroviral therapy (ART) has been available in Angola since 1996 for those who could buy antiretroviral drugs in the black market or abroad. In January 2008, the estimated number of people on ART in Angola was 11240, 25% of the estimated adults in need of treatment [571]. According to a recent retrospective study, the most frequently used triple therapy regimens include 3TC, AZT and NVP or 3TC, d4T and NVP [572]. In this study, drug resistance mutations, mostly M184V and K103N, were detected in 78% of the patients undergoing therapy for ≥ 6 months. This high rate of resistance suggests that some of the patients might have been initially infected with drug resistant strains. There is, however, no data regarding transmitted drug resistance in Angola. Hence, this work was set up to investigate, for the first time, the prevalence of transmitted HIV-1 drug resistance in several provinces of Angola.

Blood samples were collected in 2001 from 196 HIV-positive drug naive subjects living in Benguela (4 patients, 2%), Cabinda (26, 13.3%), Cuanza Norte (1, 0.5%), Cuanza Sul (1, 0.5%), Luanda (150, 76.5%), Lunda Norte (4, 2%), Malange (2, 1%), Uíge (1, 0.5%) and Zaire (3, 1.5%). The epidemiological, clinical and virological characterization of the patients is described in Table 3.1. The study was reviewed and approved by the Ethics Committees of the participating institutions.
Plasma viral load was determined with the Abbott RealTime HIV-1 assay (Abbott Laboratories, USA) [573]. *Pol* (PR and/or RT) gene sequences were obtained using an in-house method described elsewhere [570]. DNA sequences were obtained with Big Dye Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems, USA) and the 3100-Avant Genetic Analyzer (Applied Biosystems, USA). Sequences have been assigned the following GenBank accession numbers, EU031840-EU031891 and EU068199-EU068462. Viruses were genotyped using phylogenetic analyses, as described previously [570]. Resistance mutation analysis was performed using the Stanford Genotypic Resistance Interpretation Algorithm (http://hivdb6.stanford.edu/). Mutations specifically associated with transmitted HIV drug resistance were selected from two recently published lists [574, 575].

The amplification and sequencing of the PR and/or RT regions were completed successfully for 152 (77.6%) patients (Table 3.1). Phylogenetic analysis revealed that 89 (58.6%) viral isolates had concordant subtype classification in the PR and RT regions; 61 (40.1%) isolates were recombinants composed of different subtypes in these two regions, and two isolates (1.3%) were unclassifiable (Table 3.1). The following pure subtypes and sub-subtypes were identified: A1 (13, 8.6%), A2 (9, 5.9%), A3 (1, 0.6%), C (19, 12.5%), D (8, 5.3%), F1 (15, 9.9%), G (10, 6.6%), H (9, 5.9%) and J (5, 3.3%). The most prevalent recombinant strains were CRF02_AG (5, 3.3%), G/H (4, 2.6%) and U/H (4, 2.6%).

TABLE 3.1

Comparison of Demographic, Immunologic and Virologic Characteristics Between HIV Infected Patients Analyzed in This Study

Variable[a]	Value for sample group			*p*[b]
	Total	Unsequenced	Sequenced	
No. of patients (%)	196	44 (22.4)	152 (77.6)	-
Mean age (yr) (SD)	32 (10.9)	27 (9.7)	34 (10.8)	**0.0005**[**]
No. of patients by gender (%)				
Male	77 (39.3)	22 (50)	58 (38)	0.2926[#]
Female	104 (53.1)	21 (48)	83 (55)	
Unknown	15 (7.6)	1 (2)	11 (7)	-
No. of patients by transmission route(%)				
Sexual	142 (72.4)	30 (68.2)	112 (73.7)	0.5656[#]
Heterosexual	135 (68.9)	29 (65.9)	106 (69.7)	0.7120[#]
MSM	1 (0.5)	-	1 (0.7)	-
Bisexual	6 (3)	1 (2.3)	5 (3.3)	1.0000[#]
Parenteral	7 (3.5)	5 (11.4)	2 (1.4)	**0.0069[#]**
Blood transfusion	4 (2)	3 (6.8)	1 (0.7)	**0.0360[#]**
IDU	3 (1,5)	2 (4.6)	1 (0.7)	0.1271[#]
Vertical	8 (4.1)	4 (9)	4 (2.6)	0.0773[#]
Unknown	39 (20)	5 (11.4)	34 (22.3)	0.1345[#]
CDC clinical stage				
A	38 (19.4)	9 (20.5)	29 (19.1)	0.8307[#]
B	69 (35.2)	18 (41)	51 (33.6)	0.3758[#]
C	42 (21.4)	12 (27.1)	30 (19.7)	0.3005[#]
Unknown	47 (24)	5 (11.4)	42 (27.6)	**0.0276[#]**
Mean log no. of HIV RNA copies /ml (SD)	5.4 (0.59)	5.5 (0.49)	5.4 (0.67)	0.8268[#]
No. of isolates (%)				
Pure subtype			89 (58.6)	
Recombinant[*]			61 (40.1)	
Untypable			2 (1.3%)	

[a] MSM, men who have sex with men; IDU, intravenous drug user; *, different subtype classifications in PR and RT.

[b] *P* values are based on comparision of unsequenced and sequenced samples. Bold indicates statistical significance ($p < 0.05$). **, Mann-Withney U test; #, Fisher exact test.

Plasma viral load could be determined in all 35 specimens that were tested. Sequences were obtained for 21 (60%) of these patients and belonged to the following subtypes: A1 (1 isolate), A2 (1), C (2), D (1), F1 (5), G (1), H (4), J (1), A1/J (1), A1/ CRF02_AG (1), G/A1 (1), G/H (1) and CRF05_DF/D (1). These results provide definitive evidence that the Abbott RealTime HIV-1 assay is particularly well suited for viral load quantification in countries with highly complex and divergent HIV strains [573]. Consistent with the lack of antiretroviral therapy, viral load was high in most patients (mean 5.4 log copies/ml; standard deviation [SD], 0.59). There was a positive correlation between age of the patients and viral load (Spearman r = 0.4543; *P* = 0.0386).

The minor protease inhibitor (PI) resistance mutations L10I, L10V, V11I and T74P, were detected in some Angolan isolates (see Table S3.2 in the supplemental material). L10I and L10V, associated with resistance to most of the PIs when present with other mutations [485, 576], were found in 17.7% of the isolates. This is higher than the frequencies previously described in untreated patients (5-10%) (http://hivdb6.stanford.edu/pages/asi/). V11I, associated with resistance to darunavir [485, 486], was detected in 4.3% of the Angolan patients, all from subtype A. This frequency is significantly higher than that found in sequences from the same subtype available in the Stanford database. T74P was found in one patient (0.7%). T74P is one of 11 darunavir minor mutations [486] and was considered a major tipranavir resistance mutation in the RESIST study [576]. These results suggest that some Angolan isolates might have a low genetic barrier to resistance to some PIs.

Two out of 122 (1.6%) patients harboured nucleoside RT inhibitor- and nonnucleoside RT inhibitor-resistant viruses. The nucleoside RT inhibitor resistance mutations M41L, D67N, M184V, L210W and T215Y, were detected in one patient (01AOHDC232, subtype G); T215F was detected in patient 01AOHAB83 (F1/C recombinant). The former isolate also carried the K103N mutation that confers resistance to nevirapine and efavirenz. The origin of these isolates was Luanda (01AOHAB83) and Cabinda (01AOHDC232). These mutations are among the most common transmitted drug resistance mutations reported worldwide [574, 575, 577-579]. These two isolates should not be fully sensitive to the standard first-line antiretroviral regimens currently used in Angola and most other resource limited settings [572, 580, 581]. According to the WHO criteria [582], Angola has a low prevalence of transmitted resistance that is similar to other Sub-Saharan African countries where ART is still not widely available [254, 258, 259, 511, 577, 583, 584]. Nonetheless, the finding of transmitted HIV-1 drug resistance in Angola was unexpected due to the restricted availability of antiretroviral drugs until 2001. The two most likely explanations for the origin of the resistant isolates are (i) the unregulated and unmonitored use of antiretroviral drugs bought in the black market or abroad [585] and (ii) displacements of people from countries where ART is available for a longer period of time [583]. In this context, it is not surprising that transmitted HIV-1 resistance has been first detected in Cabinda and Luanda since the oil business as always attracted an important number of migrant workers to Cabinda and escape from the civil war, ending in 2002, has brought many of war refugees to Luanda.

The level of genetic diversity among the Angolan isolates was significantly higher than the isolates (same or different subtype) present in the Stanford Database. For instance, the frequency of the PR polymorphisms found in Angolan isolates of subtypes A (80% of polymorphisms), C (23%), D (50%), F (61.5%), G (12.5%), and CRF02_AG (10%) was significantly different from those found in isolates from worldwide treatment-naive patients infected with the same subtypes (see Table S3.2 in the supplemental material) (http://hivdb6.stanford.edu/pages/asi). Likewise, the frequency of the RT polymorphisms found in subtypes A (46%), C (31%), D (14%), F (34.8%), G (34.6%) and CRF02_AG (37.5%) were significantly different from those found in isolates from worldwide treatment-naive patients infected with the same subtypes (see Table S3.3 in the supplemental material).

Finally, we found some new polymorphisms in Angolan viruses that have not been described previously in the Stanford database for untreated patients. In the PR, this is the case for N37I in subtype F, T74P in subtype G and H69A in CRF02_AG (see Table S3.2 in the supplemental material). In the RT, V35Q, Q174V, V245G (subtype A), D121F (subtype D), and A272S (subtype F) are all new polymorphisms (see Table S3.3 in the supplemental material). Elucidation of the extent to which these polymorphisms may impact ART therapy requires further studies.

In conclusion, our results show an unprecedented level of genetic diversity in the PR and RT proteins of HIV-1 isolates circulating in Angola. Future studies are needed to assess the impact of this diversity on ART and resistance development. Our results also indicate that drug resistant HIV-1 strains were already being transmitted in 2001 in Angola. These results are particularly important in view of the recent increase in antiretroviral therapy programs in Angola [571]. Overall, the low prevalence of transmitted HIV drug resistance found here indicates that simplified first-line regimens can be successfully used in the vast majority of HIV-1 patients from Angola. Continued surveillance of drug resistance in treated and untreated populations will be important to maximize the efficacy of antiretroviral therapy in Angola.

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Supplemental Material

TABLE S3.2

Minor Mutations and Natural Polymorphisms Detected in the Protease of Drug-naive Patients from Angola

Codon	Patients (%)																						
	Angola (n=141)										World *						P value (Angola vs World)#						
	A1 (n = 20)	A2 (n = 13)	(n = 8)	C (n = 22)	D (n = 6)	F1 (n = 15)	G (n = 18)	H (n = 9)	J (n = 5)	02AG (n = 12)	A (n = 1524)	C (n = 2145)	D (n = 512)	F (n = 605)	G (n = 619)	02AG (n = 1415)	A	C	D	F	G	02AG	Non-B_{AO} vs B_W
L10I	50.0	-	37.5	-	-	6.7	27.8	11.1	20.0	25.0	6.9	-	-	9.9	8.2	4.6	<0.0001	-	-	1	0.0155	0.0167	<0.0001
L10V	35.0	7.7	-	4.5	-	86.7	5.6	-	-	8.3	4.1	1.1	-	19	1.2	9.4	0.0003	0.6213	-	<0.0001	<0.0001	1	<0.0001
V11I	5.0	-	62.5	-	-	-	-	-	-	-	0.3	-	-	-	-	-	<0.0001	-	-	-	-	-	<0.0001
T74P	-	-	-	-	-	-	5.6	-	-	-	-	-	-	-	-	-	-	-	-	-	0.0283	-	0.0004
T12S	-	-	-	72.7	-	-	-	-	-	-	-	65.0	-	-	-	-	-	0.5942	-	-	-	-	<0.0001
I13A	-	-	-	-	-	-	-	-	-	8.3	-	-	-	-	-	6.6	-	-	-	-	-	0.56	0.0004
I13V	100	-	100	-	-	73.3	100	100	-	91.7	89	-	-	12.0	98	92.0	0.0004	-	-	<0.0001	1	1	<0.0001
K14Q	-	-	-	-	-	6.7	-	-	-	-	-	-	-	0.2	-	-	-	-	-	0.0478	-	-	0.0004
K14R	-	-	-	-	-	-	77.8	-	-	-	-	-	-	-	69	-	-	-	-	-	0.6055	-	0.4284
I15V	40.0	-	-	95.5	-	73.3	-	-	60.0	-	6.7	85.0	-	79.0	-	-	0.0062	0.2844	-	0.5337	-	-	<0.0001
G16E	40.0	61.5	-	-	-	86.7	-	66.7	-	-	11.0	-	-	16.0	-	-	<0.0001	-	-	<0.0001	-	-	<0.0001
L19I	-	-	-	59.1	-	-	-	-	-	-	-	60.0	-	-	-	-	-	0.8949	-	-	-	-	0.2867
L19V	-	-	-	22.7	-	-	-	-	-	-	-	10.0	-	-	-	-	-	0.1078	-	-	-	-	0.053
K20I	-	-	100	-	-	-	94.4	-	-	91.7	3.3	-	-	-	98.0	93.0	<0.0001	-	-	-	0.3136	0.5833	<0.0001
K20R	-	-	-	-	-	60.0	-	77.8	-	-	-	-	-	27.0	-	-	-	-	-	0.0081	-	-	<0.0001
K20V	-	-	-	-	-	-	-	-	-	8.3	-	-	-	-	-	1.0	-	-	-	-	-	0.1195	0.0004
E35D	7.0	76.9	75	-	-	93.3	-	-	60	-	95	-	-	87.0	-	-	<0.0001	-	-	0.7062	-	-	0.011
E35N	-	23.1	-	-	-	-	-	-	-	-	1.3	-	-	-	-	-	0.0205	-	-	-	-	-	<0.0001

TABLE S3.2 *(continued)*

Codon	Patients (%)																						
	Angola (n=141)										World *						P value (Angola vs World)#						
	A1 (n = 20)	A2 (n = 13)	(n = 8)	C (n = 22)	D (n = 6)	F1 (n = 15)	G (n = 18)	H (n = 9)	J (n = 5)	02AG (n = 12)	A (n = 1524)	C (n = 2145)	D (n = 512)	F (n = 605)	G (n = 619)	02AG (n = 1415)	A	C	D	F	G	02AG	Non-B_{AO} vs B_W
M36I	18.0	100	100	72.7	-	100	100	88.9	100	100	98.0	81.0	-	92.0	98.0	97.0	0.2035	0.4796	-	0.6203	1	1	<0.0001
M36L	-	-	-	27.2	-	-	-	-	-	-	-	3.6	-	-	-	-	-	<0.0001	-	-	-	-	0.5521
N37D	7.0	30.8	-	-	-	6.7	-	-	60.0	-	9.7	4.0	-	-	-	-	0.0018	<0.0001	-	-	-	-	0.9149
N37E	-	-	-	-	-	-	-	100	40.0	-	-	-	-	-	-	-	-	-	-	-	-	-	0.4195
N37I	-	-	-	-	-	6.7	-	-	-	-	-	-	-	-	-	-	-	-	-	0.0242	-	-	0.3597
N37S	-	-	-	63.6	-	-	-	-	-	-	-	9.0	-	-	-	-	-	<0.0001	-	-	-	-	0.0662
R41K	18.0	76.9	75	86.3	100	100	100	77.8	100	100	97.0	80.0	96.0	89.0	96.0	94.0	<0.0001	0.6347	1	0.3888	1	1	<0.0001
R57K	-	-	-	-	-	93.3	-	-	-	-	-	-	-	82.0	-	-	-	-	-	0.4901	-	-	0.5278
D60E	-	-	-	-	-	-	-	88.9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.6247
D60N	-	-	-	-	-	-	-	-	60.0	-	-	-	-	-	-	-	-	-	-	-	-	-	<0.0001
Q61D	-	-	-	-	-	-	-	-	80.0	-	-	-	-	-	-	-	-	-	-	-	-	-	0.0003
I62V	-	-	-	-	-	-	-	100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	<0.0001
L63P	-	-	-	-	50.0	-	-	-	-	-	-	-	29.0	-	-	-	-	-	0.0002	-	-	-	0.2338
L63T	-	-	-	-	-	80.0	-	-	-	-	-	-	-	2.5	-	-	-	-	-	<0.0001	-	-	0.1451
L63V	35.0	-	-	-	-	-	-	-	-	-	1.8	-	-	-	-	-	<0.0001	-	-	-	-	-	<0.0001
E65D	-	-	-	-	-	60.0	-	-	-	-	-	-	-	0.3	-	-	-	-	-	<0.0001	-	-	0.0088
C67E	-	-	-	-	-	-	55.6	-	100	-	-	-	-	-	17.0	-	-	-	-	-	0.0003	-	<0.0001
H69A	-	-	-	-	-	-	-	-	-	8.3	-	-	-	-	-	0	-	-	-	-	-	0.0084	0.0004
H69K	75.0	100	87.5	100	-	-	94.4	100	100	83.3	97.0	98.0	-	-	96.0	97.0	0.0018	0.9223	-	-	0.5326	0.0504	<0.0001
H69Q	25.0	-	12.5	-	-	-	4.5	-	-	8.3	1.1	-	-	-	1.2	1.1	<0.0001	-	-	-	0.2059	0.1344	0.0501
V77I	20.0	-	-	-	-	-	-	-	-	-	21	-	-	-	-	-	0.1151	-	-	-	-	-	<0.0001
V82I	-	-	-	-	-	-	77.8	-	-	-	-	-	-	-	86.0	-	-	-	-	-	0.3078	-	<0.0001
L89I	50.0	-	-	9.1	-	-	-	100	-	-	0.5	2.1	-	-	-	-	0.0264	0.1324	-	-	-	-	<0.0001

TABLE S3.2 *(continued)*

Codon	Patients (%)																						
	Angola (n=141)										World *						P value (Angola vs World)#						
	A1 (n = 20)	A2 (n = 13)	(n = 8)	C (n = 22)	D (n = 6)	F1 (n = 15)	G (n = 18)	H (n = 9)	J (n = 5)	02AG (n = 12)	A (n = 1524)	C (n = 2145)	D (n = 512)	F (n = 605)	G (n = 619)	02AG (n = 1415)	A	C	D	F	G	02AG	Non-B_{AO} vs B_W
L89M	90.0	100	100	90.9	-	100	100	-	60.0	100	98.0	84.0	-	44.0	98.0	96.0	0.2035	0.5571	-	<0.0001	1	1	<0.0001
I93L	-	-	-	95.5	-	-	-	-	60.0	-	-	94.0	-	-	-	-	-	0.8708	-	-	-	-	0.0002

*According to the Stanford HIV Drug Resistance Database; #Fisher exact test; Minor mutations are in bold letters.

Table S3.3

Natural Polymorphisms Detected in the Reverse Transcriptase of Drug-naive Patients from Angola

Codon	Patients (%)																				
	Angola (n = 122)									World*						*P* value (Angola vs World)#					
	A1	A2	C	D	F1	G	H	J	02AG	A_W	C_W	D_W	F_W	G_W	$02AG_W$						
	(n = 19)	(n = 5)	(n = 20)	(n = 7)	(n = 13)	(n = 9)	(n = 20)	(n = 6)	(n = 15)	(n = 1301)	(n = 1979)	(n = 320)	(n = 262)	(n = 398)	(n = 1025)	A	C	D	F	G	02AG	Non-B_{AO} vs B_W
V35K	5.3	-	15.0	-	7.7	-	-	-	-	0.2	2.9	-	1.3	-	-	0.0734	0.0204	-	0.177	-	-	<0.0001
V35I	-	-	-	-	-	11.1	-	-	-	-	-	-	-	2.2	-	-	-	-	-	0.2024	-	<0.0001
V35Q	-	20.0	-	-	-	-	-	-	-	-	-	-	-	-	-	0.0189	-	-	-	-	-	0.001
V35T	94.7	80.0	85.0	100	92.3	88.8	100	83.3	93.3	88.0	93.0	88.0	89.0	94.0	89.0	0.5314	0.1449	1	1	0.4381	1	<0.0001
E36A	-	-	80.0	-	-	-	-	-	-	3.3	77.0	-	-	-	-	0.223	1	-	-	-	-	<0.0001
E36Q	-	-	-	-	-	-	-	50.0	-	-	-	-	-	-	-	-	-	-	-	-	-	<0.0001
T39A	-	-	-	-	-	-	-	66.7	-	-	-	-	-	-	-	-	-	-	-	-	-	0.6803
T39E	-	-	95.0	-	-	-	-	-	-	-	70.0	-	-	-	-	-	0.0125	-	-	-	-	<0.0001
T39K	57.9	60.0	-	-	-	-	-	-	-	38.0	-	-	-	-	-	0.0945	-	-	-	-	-	<0.0001
T39L	-	-	-	-	-	-	-	16.7	-	-	-	-	-	-	-	-	-	-	-	-	-	0.3632
S48T	-	-	85.0	-	-	-	-	-	-	-	87.0	-	-	-	-	-	0.7374	-	-	-	-	<0.0001
K49R	-	-	-	85.7	-	-	50.0	-	-	-	-	76.0	-	-	-	-	-	1	-	-	-	<0.0001
V60I	-	-	-	100	-	88.8	90.0	-	73.3	-	-	94.0	-	93.0	35.0	-	-	1	-	0.3598	0.0042	<0.0001
D121C	-	-	-	42.9	-	-	-	-	-	-	-	5.4	-	-	-	-	-	0.0068	-	-	-	<0.0001
D121F	-	-	-	14.3	-	-	-	-	-	-	-	0	-	-	-	-	-	0.0214	-	-	-	0.001
D121H	-	80.0	-	-	-	-	-	-	-	7.5	-	-	-	-	-	0.1197	-	-	-	-	-	0.8772
D121Y	-	-	-	42.9	-	-	-	-	-	-	-	41.0	-	-	-	-	-	1	-	-	-	0.9081
K122E	94.7	100	75.0	100	-	77.8	85.0	83.3	60.0	91.0	91.0	83.0	-	85.0	34.0	0.7191	0.0303	0.6049	-	0.6319	0.0061	<0.0001
K122F	-	-	-	-	-	-	-	16.7	-	-	-	-	-	-	-	-	-	-	-	-	-	0.001
K122P	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.6605
K123E	-	-	5.0	-	61.5	-	-	-	-	-	2.5	-	69.0	-	-	-	0.4051	-	0.5525	-	-	<0.0001
K123G	26.3	-	15.0	-	-	-	-	-	-	6.6	25.0	-	-	-	-	0.5722	0.437	-	-	-	-	<0.0001
K123N	31.6	-	50.0	-	-	-	-	-	-	23.0	13.0	-	-	-	-	0.8151	<0.0001	-	-	-	-	<0.0001
K123S	26.3	-	20.0	-	-	-	55.0	-	-	58.0	24.0	-	-	-	-	0.0008	0.7978	-	-	-	-	<0.0001

TABLE S3.3 *(continued)*

Codon	Patients (%)																				
	Angola (n = 122)									World*						*P* value (Angola vs World)[#]						
	A1 (n = 19)	A2 (n = 5)	C (n = 20)	D (n = 7)	F1 (n = 13)	G (n = 9)	H (n = 20)	J (n = 6)	02AG (n = 15)	A_W (n = 1301)	C_W (n = 1979)	D_W (n = 320)	F_W (n = 262)	G_W (n = 398)	$02AG_W$ (n = 1025)	A	C	D	F	G	02AG	Non-B_{AO} vs B_W
I135V	-	-	-	-	61.5	-	-	-	-	1.4	-	-	19.0	-	-	0.3052	-	-	0.0014	-	-	0.5568
I142V	-	80.0	-	-	-	-	-	-	-	9.7	-	-	-	-	-	0.0922	-	-	-	-	-	0.7365
S162A	-	-	-	-	-	-	-	-	-	3.3	-	-	-	-	-	0.7166	-	-	-	-	-	0.3465
S162C	-	-	-	-	84.6	-	-	-	-	-	-	-	42.0	-	-	-	-	-	0.0032	-	-	0.0018
K173A	42.1	20.0	65.0	-	-	-	-	-	40.0	10.0	66.0	-	-	-	13.0	0.002	1	-	-	-	0.0091	<0.0001
K173I	5.3	-	-	-	-	-	-	-	26.7	3.7	-	-	-	-	8.3	0.6133	-	-	-	-	0.0329	<0.0001
K173L	5.3	20.0	-	-	-	-	-	-	-	36.0	-	-	-	-	-	1	-	-	-	-	-	<0.0001
K173R	-	-	10.0	-	-	-	-	-	-	-	0.2	-	-	-	-	-	0.0014	-	-	-	-	0.8621
K173S	42.1	40.0	-	-	-	-	-	-	13.3	44.0	-	-	-	-	2.8	1	-	-	-	-	0.071	<0.0001
K173T	5.3	-	25.0	-	-	-	-	50	13.3	2.7	22	-	-	-	66.0	0.5007	0.786	-	-	-	<0.0001	<0.0001
Q174E	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.2743
Q174H	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.8854
Q174K	57.9	-	-	-	100	77.8	-	66.7	53.3	76.0	-	-	91.0	85.0	68.0	0.0007	-	-	0.6127	0.6319	0.2671	<0.0001
Q174L	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.631
Q174V	-	80.0	-	-	-	-	-	-	-	-	-	-	-	-	-	<0.0001	-	-	-	-	-	<0.0001
D177E	73.7	100	90.0	100	-	100	90.0	-	60.0	86.0	79.0	94.0	-	89.0	93.0	0.3825	0.8003	1	-	0.606	0.0004	<0.0001
I178V	-	60.0	-	-	-	-	-	-	-	2.1	-	-	-	-	-	0.0172	-	-	-	-	-	0.0713
G196E	-	-	-	-	-	-	-	100	-	5.1	-	-	-	-	-	1	-	-	-	-	-	0.0131
T200A	73.7	100	95.0	57.1	84.6	100	50.0	-	73.3	27.0	91.0	12.0	49.0	90.0	89.0	0.0039	<0.0001	0.0056	0.0196	0.6086	0.0779	<0.0001
T200E	-	-	5.0	-	15.4	-	-	16.7	-	-	2.1	-	3.3	-	-	-	0.354	-	0.0616	-	-	0.3038
T200I	-	-	-	-	-	-	-	50	-	-	-	-	-	-	-	-	-	-	-	-	-	0.0351
T200K	-	-	-	-	-	-	-	16.7	-	-	-	-	-	-	-	-	-	-	-	-	-	0.8554
T200M	-	-	-	-	-	-	-	16.7	-	-	-	-	-	-	-	-	-	-	-	-	-	0.3632
T200V	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.995

TABLE S3.3 *(continued)*

Codon	Patients (%)																				
	Angola (n = 122)									World*						*P* value (Angola vs World)[#]						
	A1 (n = 19)	A2 (n = 5)	C (n = 20)	D (n = 7)	F1 (n = 13)	G (n = 9)	H (n = 20)	J (n = 6)	02AG (n = 15)	A_W (n = 1301)	C_W (n = 1979)	D_W (n = 320)	F_W (n = 262)	G_W (n = 398)	$02AG_W$ (n = 1025)	A	C	D	F	G	02AG	Non-B_{AO} vs B_W
Q207A	52.6	80.0	25.0	-	7.7	11.1	70.0	16.7	33.3	91.0	7.7	-	30.0	3.7	8.6	<0.0001	0.0165	-	0.1171	0.3055	0.0075	<0.0001
Q207D	5.3	-	-	-	42.3	-	5.0	-	-	2.2	-	-	3.0	-	-	0.4386	-	-	0.0008	-	-	0.0006
Q207E	21.1	20.0	65.0	100	46.2	55.6	-	83.3	33.3	3.8	64	67.0	46.0	19.0	73.0	0.0026	1	0.1009	1	0.018	0.0017	<0.0001
Q207G	15.8	-	-	-	7.7	22.2	-	-	13.3	0.4	-	-	2.6	1.6	3.7	0.0003	-	-	0.3247	0.0114	0.1105	<0.0001
Q207K	-	-	-	-	-	11.1	-	-	13.3	-	-	-	-	57.0	2.6	-	-	-	-	0.0122	0.063	0.561
Q207N	5.3	-	-	-	15.4	-	5.0	-	-	0.8	-	-	1.7	-	-	0.1896	-	-	0.0294	-	-	0.1336
Q207R	-	-	5.0	-	-	-	-	-	6.7	-	0.8	-	-	-	0.3	-	0.1577	-	-	-	0.0565	0.7159
Q207T	-	-	-	-	-	-	20.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	<0.0001
R211K	47.4	80.0	45.0	57.1	53.8	55.6	85.0	50.0	-	9.1	63	78.0	77.0	64.0	-	<0.0001	0.1073	0.1883	0.0885	0.7282	-	0.0486
R211N	10.5	-	-	-	-	-	-	-	-	4.1	-	-	-	-	-	0.278	-	-	-	-	-	0.0197
R211S	36.8	-	-	-	-	-	-	-	-	79	-	-	-	-	-	<0.0001	-	-	-	-	-	0.0002
I244V	-	-	-	-	-	-	95.0	50.0	-	-	-	-	-	-	-	-	-	-	-	-	-	<0.0001
V245E	5.3	-	-	-	-	11.1	5.0	-	6.7	15	-	-	-	0.9	10.0	0.159	-	-	-	<0.0001	1	0.0104
V245G	5.3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.0189	-	-	-	-	-	0.3632
V245K	31.6	60.0	5.0	-	-	11.1	80.0	-	6.7	9.5	7.2	-	-	1.2	8.8	0.0004	0.9025	-	-	0.1263	1	<0.0001
V245L	-	-	-	-	-	-	-	-	6.7	-	-	-	-	-	1.6	-	-	-	-	-	-	0.6978
V245Q	52.6	40.0	90.0	-	100	77.8	5.0	100	80.0	41.0	82.0	-	98.0	97.0	72.0	0.3071	0.5574	-	1	0.0345	0.772	<0.0001
V245T	-	-	-	-	-	-	10.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.8636
E248D	-	-	-	-	69.2	-	-	83.3	-	31.0	-	-	59.0	-	-	0.0017	-	-	0.5709	-	-	<0.0001
E248N	-	-	-	-	-	-	-	16.7	-	-	-	-	-	-	-	-	-	-	-	-	-	0.995
E248T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.001
D250E	-	-	35.0	-	-	55.6	50.0	66.7	-	-	16.0	-	-	94.0	-	-	0.0317	-	-	0.0018	-	<0.0001
A272G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.6282
A272P	52.6	-	65.0	100	69.2	66.7	50.0	83.3	53.3	33.0	80.0	77.0	100	96.0	22.0	0.2842	0.1548	0.357	<0.0001	0.0061	0.0086	0.0263

TABLE S3.3 *(continued)*

Codon	Patients (%)																				
	Angola (n = 122)									World*						*P* value (Angola vs World)#					
	A1	A2	C	D	F1	G	H	J	02AG	A_W	C_W	D_W	F_W	G_W	$02AG_W$						
	(n = 19)	(n = 5)	(n = 20)	(n = 7)	(n = 13)	(n = 9)	(n = 20)	(n = 6)	(n = 15)	(n = 1301)	(n = 1979)	(n = 320)	(n = 262)	(n = 398)	(n = 1025)	A	C	D	F	G	02AG	Non-B_{AO} vs B_W
A272Q	5.3	-	-	-	-	-	-	-	-	0.5	-	-	-	-	-	0.1416	-	-	-	-	-	0.001
A272S	26.3	-	-	-	30.8	-	-	-	-	3.1	-	-	-	-	-	0.0011	-	-	<0.0001	-	-	0.8085
K277R	-	-	55.0	42.9	-	-	-	-	-	-	64.0	65.0	-	-	-	-	0.4835	0.0748	-	-	-	<0.0001
K281R	-	100	-	-	-	-	-	-	-	22.0	-	-	-	-	-	1	-	-	-	-	-	0.6069
T286A	73.7	100	55.0	71.4	100	-	90.0	50	-	85.0	73.0	72.0	80.0	-	-	0.569	0.0798	1	0.1376	-	-	<0.0001
E291D	63.2	100	95.0	-	100	77.8	95.0	83.3	86.7	91.0	95.0	-	96.0	90.0	92.0	0.0061	1	-	1	0.2354	0.345	<0.0001
E292I	-	80.0	85.0	-	100	77.8	90.0	66.7	93.3	67.0	94.0	-	87.0	94.0	86.0	<0.0001	0.1185	-	0.3802	0.1071	0.7079	<0.0001
V292T	-	-	-	-	-	-	-	-	6.7	-	-	-	-	-	0.4	-	-	-	-	-	0.0702	0.001
I293V	100	100	90.0	-	100	100	95.0	100	100	98.0	94.0	-	97.0	96.0	99.0	1	0.3438	-	1	1	1	<0.0001
P294T	-	-	-	-	-	-	-	66.7	-	70.0	-	-	-	-	-	<0.0001	-	-	-	-	-	0.0493
E297A	-	20.0	-	-	92.3	88.9	-	83.3	-	0.2	-	-	64.0	78.0	-	0.513	-	-	0.0387	0.6905	-	0.029
E297K	-	80.0	-	-	-	-	-	-	-	5.4	-	-	-	-	-	0.011	-	-	-	-	-	<0.0001
A304E	-	80.0	-	-	-	-	-	-	-	2.8	-	-	-	-	-	0.0057	-	-	-	-	-	0.1685
K311R	-	-	-	-	-	-	100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.9733
E312D	-	80.0	-	-	-	-	-	-	-	64.0	-	-	-	-	-	<0.0001	-	-	-	-	-	<0.0001
P321S	-	-	-	-	-	-	-	60.0	-	-	-	-	-	-	-	-	-	-	-	-	-	0.0889
S322T	-	-	-	-	-	-	-	50.0	-	-	-	-	-	-	-	-	-	-	-	-	-	0.0403
D324E	-	-	-	-	-	55.6	-	83.3	-	-	-	-	-	83.0	-	-	-	-	-	0.0561	-	0.003
I326V	52.6	-	-	-	-	-	-	-	40.0	11.0	-	-	-	-	62.0	<0.0001	-	-	-	-	0.1073	0.5677
I329V	-	-	-	-	-	66.7	-	66.7	-	-	-	-	-	91.0	-	-	-	-	-	0.0456	-	0.9129
Q334L	-	-	-	-	-	66.7	-	66.7	-	-	-	-	-	51.0	-	-	-	-	-	0.5044	-	0.0272
G335D	84.2	-	60.0	-	-	88.9	55.0	50.0	73.3	91.0	86.0	-	-	38.0	89.0	0.0014	0.0041	-	-	0.0029	0.0779	<0.0001
G335E	-	-	-	-	-	11.1	-	-	-	-	-	-	-	0.8	-	-	-	-	-	0.0859	-	0.3632

*According to the Stanford HIV Drug Resistance Database; #Fisher exact test.

CHAPTER 4

HIV genetic diversity and transmitted drug resistance in health care settings in Maputo, Mozambique

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Abstract

Objectives: To characterize HIV-1 diversity and transmitted drug resistance in Maputo, Mozambique in persons with access to care and treatment in Maputo, Mozambique.

Methods: Samples were collected in 2002–2004 from 144 drug naive patients attending public hospitals and private clinics. Plasma viremia, CD4, and CD8 cell counts were determined for each patient. The Stanford Algorithm was used for resistance genotyping on *pol* sequences. Subtyping was done by phylogenetic analysis.

Results: Most patients had high viral load (mean, 5.0 log copies/mL) and low CD4 cell counts (median, 260 CD4 cells/mL). Protease and/or reverse transcriptase sequences were obtained from 104 (72%) samples. Patients harbored subtypes C (80.8%), G (3.8%), CRF37_cpx (6.7%), untypable (U) (1.0%), and recombinant strains (7.7%) comprising the A, C, D, F, and U clades. There were no major protease inhibitor resistance mutations. Mutations conferring resistance to the nucleoside/nucleotide reverse transcriptase inhibitors and/or nonnucleoside reverse transcriptase inhibitors were found in 4 (4/68; 5.9%) patients. Phylogenetic analysis suggested an imported origin for 2 resistant variants.

Conclusions: The HIV-1 epidemic in Maputo is evolving rapidly in genetic complexity due to the recent introduction of all major subtypes and recombinant forms. Continued surveillance of drug resistance in treated and untreated populations is needed to prevent further transmission of HIV drug-resistant variants and maximize the efficacy of antiretroviral therapy in Maputo.

Introduction

HIV was introduced into Mozambique probably in the early 1980s and since than it has spread at a very high rate, especially among women [586, 587]. As of end 2007, there were more than 1.8 million individuals living with HIV/AIDS in Mozambique and the estimated adult HIV prevalence rate in the country was 16% (14-17%) [588]. HIV-1 infection reaches higher prevalence rates in the most populated Southern region (21%, range 16-23%) which include Maputo Province (26%, 18-34%), Maputo City (23%, 18-29%), Gaza (27%, 18-35%) and Inhambane (12%, 7-16%) [588].

The introduction of HAART into developed countries in 1996 has caused a significant decrease in the mortality and morbidity rates associated with HIV-1 infection and the prognosis for patients who have access to these drugs has also improved significantly [12-14]. As of 2004, simplified antiretroviral (ART) regimens are freely provided to an increasing number of HIV infected patients from developing countries with similar virological, immunological and clinical benefits [589-595]. Most of these countries are using the World Health Organization (WHO) public health approach to ART delivery [580]. This approach includes standard first-line regimens consisting of 2 nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs) plus a non-nucleoside reverse transcriptase inhibitor (NNRTI) with 1 or 2 alternate regimens based on single drug substitutions and only 1 second-line regimen based on a boosted protease inhibitor (PI) (with at least 1 NRTI), with treatment switch guided by clinical disease progression.

Widespread use of ART without the proper infrastructure to monitor patients may lead to the rapid emergence of resistant viral strains that will limit therapeutic options for patients and increase the risk of transmission of resistant strains [582, 585, 596]. In resource-limited settings, transmission of drug resistant HIV variants is a major public health concern as it could lead very rapidly to a situation in which no effective drugs are available for the treatment of HIV infection [582, 597, 598]. Several studies performed with drug naïve patients enrolled in 2001 up to 2006 from different countries have shown that transmitted HIV drug resistance is still rare (prevalence <5%) in sub-Saharan Africa [254, 257-259, 577, 583, 584].

Although undocumented, ART has been available in Maputo since 1996 for those infected with HIV who could buy antiretroviral drugs in the black market or abroad. In 2003, a national plan was developed in Mozambique to provide expanded and free access to ART using the WHO public health approach to ART delivery [580]. As of 2004, the number of people on ART has increased from < 4000 in May 2004 to 91,312 in January 2008, 32% of the estimated 285,000 adults in need of treatment. About 25,316 (28%) patients doing ART reside in the city of Maputo. Mozambique is currently implementing the WHO strategy for prevention and assessment of primary and acquired drug resistance [582]. The first primary HIV drug resistance survey will be done with available data and specimens from the 2007 round of HIV surveillance.

At this time, only 3 small studies have looked into the genetic diversity and HIV drug resistance in antiretroviral-naive patients in Mozambique. These studies were performed in Manhiça, a rural village which is located 80 km from Maputo [147]; Matola and Machava 2 main centers located in the

outskirts of Maputo [599]; and Beira, the second largest city of Mozambique, which is located in the central region of the country [600]. With two exceptions, all patients were infected with clade C virus. Evidence for transmitted HIV drug resistance was only obtained in Beira where 4 out of 43 (9.3%) patients harbored NRTI or NNRTI resistance viruses and 1 isolate was PI resistant [600].
This work was set up to investigate, for the first time, the genetic diversity and molecular epidemiology of HIV-1 in drug-naive patients from the city of Maputo and determine the prevalence of transmitted HIV drug resistance in 2002-2004.

Methods

Population

One hundred and forty four plasma samples from drug-naive HIV-1 positive individuals were collected during 2002 (N = 34; 24%), 2003 (N = 74; 51%) and 2004 (N = 36; 25%) from patients referring to 4 different hospitals in Maputo (Military Hospital of Maputo, Central Hospital of Maputo, Day Hospital of Alto Mae, Day Hospital of 1º de Maio) and 3 private clinics. The year of infection was unknown in most patients. Patients were referred to the Viral Immunodiagnostic Unit, located at the Faculty of Medicine, Eduardo Mondlane University in Maputo, to assess their immunological and virological status. Contrary to most HIV-1 infected patients in Maputo, these patients were characterized by a medium to high socioeconomic status and all could afford to pay for $CD4^+$ T-cell counts and/or plasma viral load. Some symptomatic patients were candidates to self-payed ART. Serological diagnosis of HIV infection, immune cell counts and plasma viral load were determined in Maputo. Diagnosis was done using the rapid tests Determine HIV-1/2 (Abbott) and Uni-Gold Recombigen (Trinity Biotech). The number of total lymphocytes, $CD4^+$ and $CD8^+$ T cells was determined by flow cytometric analysis using FACSCalibur (Becton Dickinson). Plasma viral load was also determined with the Cobas Amplicor HIV-1 Monitor™ test version 1.5 (Roche). The limit of detection of the assay is 400 HIV-1 RNA copies per milliliter of plasma (2.6 $\log_{10}$). Plasma samples were conserved in Maputo at -20ºC until July 2004 and thereafter in Lisbon, Portugal, at -80ºC. Resistance genotyping was performed in Lisbon. The study was reviewed and approved by the Ethics Committee of the Faculty of Medicine, Eduardo Mondlane University, Maputo, Mozambique.

Viral RNA Extraction, Polymerase Chain Reaction Amplification, and Sequencing

Viral RNA was extracted from 200 μl plasma using Nuclisens Isolation Kit (BioMerieux). Reverse transcriptase-polymerase chain reaction was performed with Titan One Tube RT-PCR System (Roche). Nested polymerase chain reaction was done to obtain a 532 base pair fragment from the protease (PR) region using outer primers IBPR1.1 and IBPR2.2 and inner primers IBPR3.1 and IB2621PR4; to obtain a 1026 bp fragment from the reverse transcriptase (RT) region we used outer primers IB2480RT1 and IB3626RT2 and inner primers IB2530RT3 and IB3555RT4. Thermal cycling

conditions for PCR and primers sequence and position were described elsewhere [167, 570]. DNA sequences were obtained with Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems) and an automated sequencer (3100-Avant Genetic Analyzer, Applied Biosystems).

Phylogenetic and Recombination Analysis

Sequences were aligned with reference strains collected from the Los Alamos Sequence Database [601] using ClustalX 1.8 [195]. Maximum-likelihood phylogenetic analyses [551] were performed using the best-fit model of molecular evolution estimated by Modeltest v3.7 under the Akaike information criterion [552]. The chosen model was GTR+I+G for both genes. Tree searches were conducted in PAUP v4.0b10 using a nearest-neighbor interchange (NNI) heuristic search strategy [553] and bootstrap resampling [554]. Bootstrap values ≥70% were considered definitive for significant clustering [555]. Recombination analysis was performed by bootscanning using SimPlot 3.5.1 [556].

Origin of Subtype G and CRF37_cpx

To understand the provenience of subtype G and CRF37_cpx viruses circulating in Mozambique, we used a maximum-likelihood phylogenetic analysis of a dataset containing our Mozambican sequences and subtype G, CRF02_AG, CRF37_cpx and A3 sequences collected from the Los Alamos Sequence Database [601]. Briefly, we used a PAUP maximum-likelihood approach to estimate the parameters of the evolutionary model from the dataset. To find the maximum-likelihood tree, an iterative heuristic method combining 2 different tree rearrangment methods was used: nearest-neighbor interchange, wich is simpler, and tree bisection and reconnection wich is the most extensive rearrangment method. This combinatory heuristic search has been previously described [602]. Bootstrap analysis and zero-branch length tests were performed to verify the statistical confidence of each cluster.

Resistance Mutation Analysis

Resistance mutation analysis was performed using the Stanford Genotypic Resistance Interpretation Algorithm [603]. All resistance mutations and unusual polymorphisms were considered in a first approach. Mutations specifically associated with transmitted HIV drug resistance were then selected from 2 recently published lists [574, 575].

Statistical Analysis

Statistical analysis was performed with GraphPad Prism version 4.0 [558]. The Spearman rank test was used to quantify the magnitude and direction of the correlation between viral load and type and number of lymphocytes. Linear regression analysis and the *F* statistics were used to discriminate between viral load and the other variables. Mann-Whitney test was used to compare groups. The

frequencies of drug resistance mutations of Mozambicans viruses were compared with those of all subtype B and C sequences available at the Stanford HIV Drug Resistance Database [603] using Fisher exact test. *P* values <0.05 were considered significant.

GenBank Accession Numbers

Sequences have been assigned the following GenBank accession numbers: DQ659965-DQ660139, EF071930-EF071942, and EU856363.

Results

Study Population

The demographic, immunologic, and virologic characteristics of the patients are described in Table 4.1. The mean age of the patients was 41 (SD, 12) years old. Fifty-seven percent of the samples were from men. Plasma viral load was high in most patients (mean 5.0 log copies/ml) and the number of T lymphocytes (median, 1275 cells/µl) and $CD4^+$ T cells (median, 260 cells/µl) was low. There was a statistically significant inverse correlation between plasma viral load and the number of T lymphocytes (Spearman r = -0.321; P < 0.0001; F = 5.4; P = 0.0215), $CD4^+$ cells (Spearman r = -0.5016; P < 0.0001; F = 56.6; P < 0.0001), CD4 percentage (Spearman r = -0.3887; P < 0.0001; F = 35.1; P < 0.0001) and CD4/CD8 ratio (Spearman r = -0.369; P < 0.0001; F = 31.8; P < 0.0001). In contrast, viral load was positively correlated with the percentage of $CD8^+$ T cells (Spearman r = 0.4133; P < 0.0001; F = 33.9; P < 0.0001). Overall, these results demonstrate that most patients were severely compromised immunologically and are perfectly consistent with the lack of antiretroviral treatment.

Plasma viremia was, however, undetectable in 12 (8%) patients. When compared to the patients with detectable plasma viremia, these patients had a significantly higher number and percentage of $CD4^+$ T cells, higher CD4/CD8 ratio, and lower percentage of CD8 cells (Table 4.1). These patients may qualify as HIV-1 elite controllers [604].

HIV-1 Genetic Diversity

The amplification, sequencing and phylogenetic analysis of the PR and/or RT regions were completed successfully for 104 (72%) samples. PR sequences were obtained from 99 (95%) patients. RT sequences were obtained from 68 (65%) patients. PR and RT sequences were obtained from 63 (61%) patients. Sequences were obtained significantly more often in older patients and patients with higher viral load (Table 4.1).

Phylogenetic analysis revealed that 84 (80.8%) viral isolates were subtype C, 12 (11.5%) were non-C [7 (58%) CRF37_cpx, 4 (33%) subtype G and 1 (8%) untypable (U) strain] and 8 (7.7%) were recombinants between the C subtype and 3 other subtypes or U strains [3 C (PR)/A1 (RT), 1 C/D, 1 C/F1, 2 C/U and 1 U/C] (Figure 4.1). Bootscanning analysis of concatenated genes confirmed the

recombination events and enabled the identification of some recombination breakpoints (data not shown).

The Mozambican C sequences were widely spread across multiple clusters containing other subtype C sequences from neighboring countries which provides evidence against a specific subtype C Mozambican cluster (Figure 4.1). Notably, 7 sequences were more closely related to each other than to any other sequences found in the databases. These sequences were closely related to CRF37_cpx and sub-subtype A3 strains from Angola (Figure 4.1C). The ML tree suggests 2 of the Mozambican subtype G strains are more closely related to Portuguese subtype G sequences, however the lack of statistical support for this clade doesn't allow us to draw any conclusions.

Drug Resistance Mutations and Other Polymorphisms

There were no major mutations associated with PI resistance. The rare minor V11I mutation, associated with darunavir resistance [485, 486], was present in all 7 patients infected with CRF37_cpx/A3-related viruses. This mutation was not found in the C sequences from Mozambique and worldwide (Table 4.2). Q58E, associated with resistance to tipranavir [576], was detected in one subtype C-infected patient. In the RT, we detected 1 or more of the following mutations, M41L, D67N, M184V, L210W, T215F, T215Y and K219Q associated with resistance to the NRTIs, in 4 patients (Table 4.3). One of the patients also carried the K103N mutation that confers resistance to nevirapine and efavirenz. Phylogenetic analysis revealed that patients 04MZUNIV87 and 04MZUNIV15 clustered together in the RT region which could be an indication of epidemiological linkage (Figure 4.1). Patients with drug resistant virus had a significantly lower viral load compared to rest of the patients suggesting a lower replicative capacity of the transmitted resistant variants (Table 4.1).

The most frequent PR polymorphisms in subtype C viruses, as compared with the B subtype, were T12S, I15V, L19I, M36I, R41K, L63P, H69K, L89M and, I93L (Table 4.2). The prevalence of the T12S, L19I and M36I mutations was significantly higher in the C sequences from Maputo compared to the worldwide C and B sequences available from untreated patients (Table 4.2). PR polymorphisms whose frequency in non-C sequences differed significantly from clade C viruses from Mozambique and worldwide were T12S, I15V, L19I, K20I, R41K and I93L.

The most frequent RT polymorphisms in subtype C viruses, as compared with the B subtype, are shown in Table 4.4. The prevalence of the V35T, D177E, V245Q, E291D and V292I polymorphisms was significantly lower in the C sequences from Maputo compared to the worldwide C and B sequences available from untreated patients (Table 4.4). In contrast, the prevalence of R211K was significantly higher in our patients. The RT polymorphisms whose frequency in non-C sequences differed significantly from clade C viruses from Mozambique and worldwide were E36A, T39E, S48T, K122E, A272P and V292I (Table 4.4).

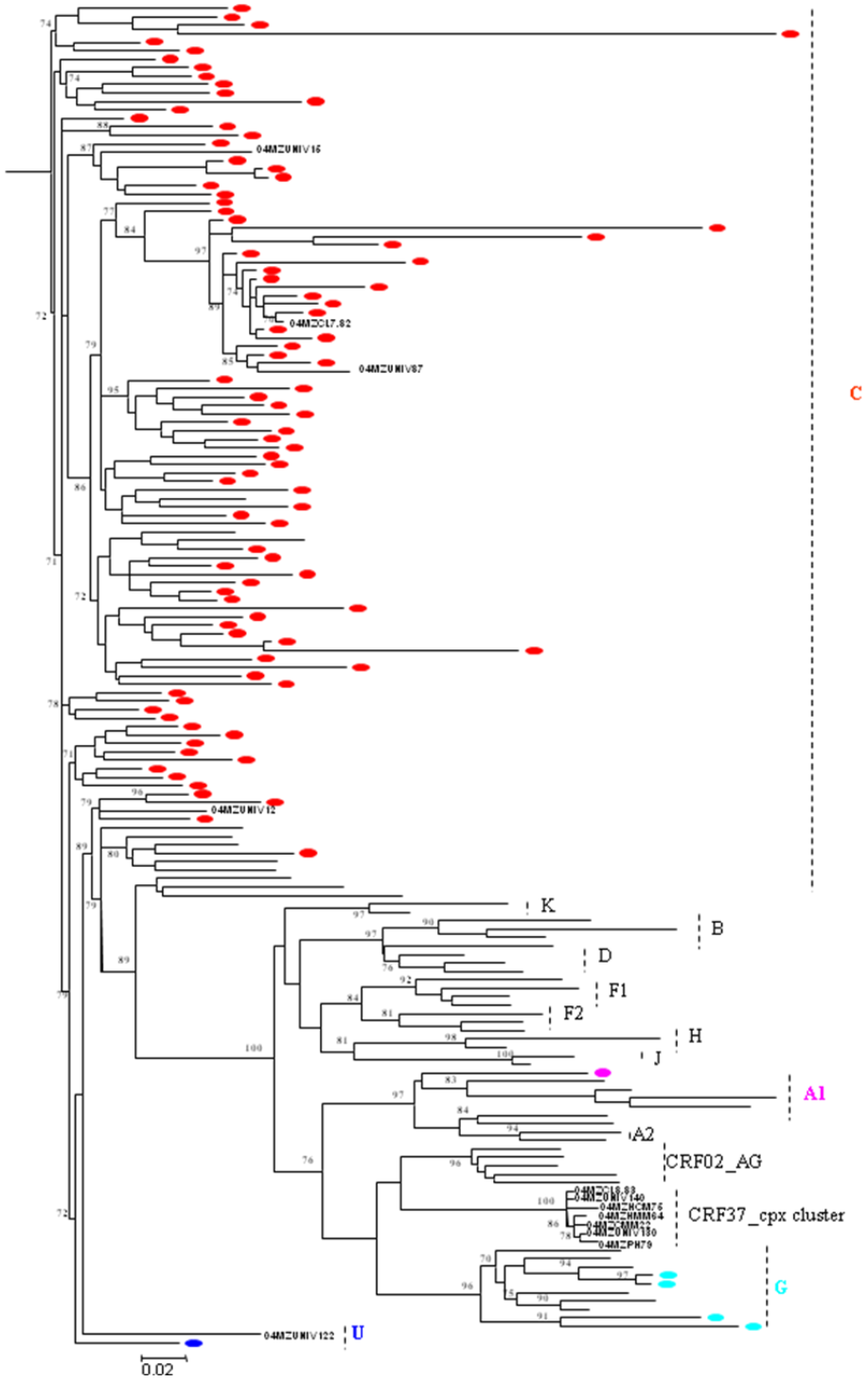
TABLE 4.1

Comparison of Demographic, Immunologic and Virologic Characteristics Between HIV Infected Patients Analysed in This Study

Variables	Total	Unsequenced samples	Sequenced samples	*P* (Unsequenced vs Sequenced)	DRM	*P* (Wilde-type[d] vs DRM patients)	DPV	UPV	*P* (DPV vs UPV)
Patients, n (%)	144	40 (28)	104 (72)	-	12 (12)	-	132 (92)	12 (8)	-
Age, mean (SD), yrs	41 (12)	38 (12)	42 (12)	**0.051**[b]	41 (10)	0.6618	41 (12)	40 (10)	0.816
Gender (%)									
Male	82 (57)	22 (55)	60 (58)	0.852[c]	8 (67)	0.7587	72 (55)	10 (83)	0.069
Female	62 (43)	18 (45)	44 (42)		4 (33)		60 (45)	2 (17)	
T-lymphocyte count median (range), cells/µl	1275 (136-8080)	1271 (368-6489)	1275 (136-8080)	0.757[b]	1509 (336-3792)	0.4146	1243 (136-8080)	1490 (536-2628)	0.218
CD4 cell count median (range), cells/µl	260 (6-1369)	355 (6-1369)	230 (7-1003)	0.1655	365 (33-1003)	0.1949	241 (6-1003)	461 (49-1369)	**0.007**
CD8 cell count median (range), cells/µl	858 (102-7280)	845 (320-5444)	858 (102-7280)	0.7651	899 (323-3211)	0.5803	858 (102-7280)	961 (327-2002)	0.980
CD4/CD8 ratio, median (range)	0.2 (0.01-2.7)	0.3 (0.01-2.7)	0.2 (0.01-2.4)	0.3444	0.4 (0.1-0.9)	0.293	0.2 (0.01-2.4)	0.5 (0.1-2.7)	**0.012**
CD4 cells percentage, median (range)	19 (1-68)	23 (1-68)	19 (1-54)	0.2706	25 (6-46)	0.2727	19 (1-54)	29 (9-68)	**0.015**
CD8 cells percentage, median (range)	75 (23-99)	70 (25-98)	76 (23-99)	0.2234	70 (51-96)	0.7373	76 (23-99)	61 (25-84)	**0.004**
HIV RNA, mean (SD), log copies/ml	5.0 (1.1)	4.4 (1.2)	5.2 (0.9)	**<0.0001**	4.3 (1.1)	**0.0041**	5.2 (0.8)	< 2.6	-
Subtype C, n (%)	-	-	84 (81)	-	2 (17)	**<0.0001**[e]	82 (79)	2 (40)	**0.048**
Non-C, n (%)	-	-	12 (12)	-	7 (58)		9 (9)	3 (60)	
Recombinant, n (%)[a]	-	-	8 (8)	-	3 (25)		8 (8)	-	-

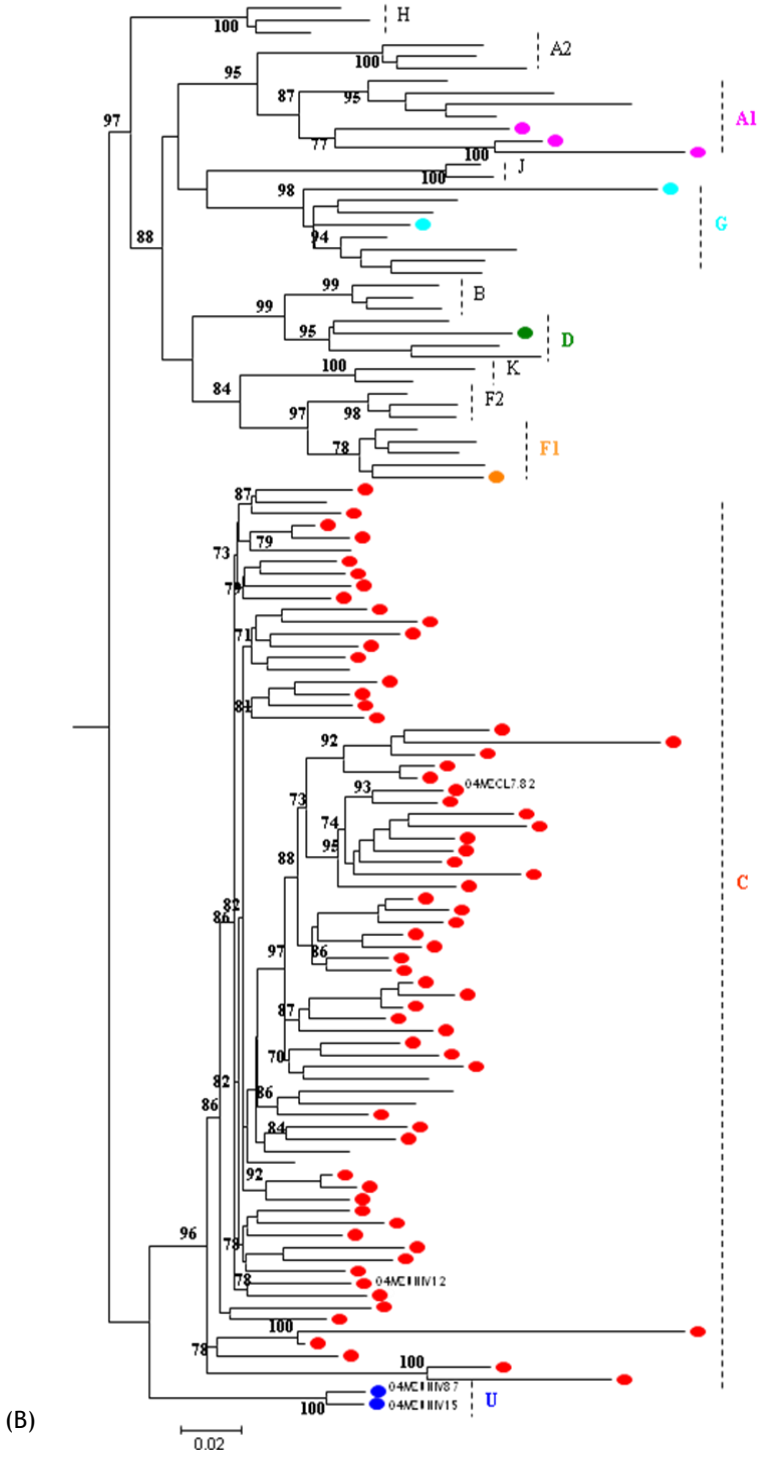
DPV, patients with detectable plasma viremia; UPV, patients with undetectable plasma viremia; DRM, patients harboring virus with mutations associated with drug resistance.

[a], different subtype classification in PR and RT; [b], Mann-Withney test; [c], Fisher exact test; [d], wild-type- patients without drug resistant mutations (n= 92); [e], comparison between subtype C and all other strains (non-C + recombinant viruses) (Fisher exact test).



(A)

FIGURE 4.1 - Genetic subtypes and evolutionary relationships of the viruses sequenced in this study based on maximum likelihood phylogenetic trees of PR (A) and RT (B) regions. The phylogenetic trees were constructed with reference sequences from all HIV-1 subtypes and sub-subtypes as well as with the Mozambican sequences (all sequences shown in colored symbols except the sequences with drug resistance mutations).



(B)

FIGURE 4.1 *(continued)* - C, Origin of the G and CRF37_cpx strains found in Mozambique. The maximum likelihood phylogenetic tree was constructed with genomic sequences from all subtype G, A3 and CRF37_cpx available in GenBank. In each tree, the bootstrap values supporting the internal branches defining a subtype or a sub-subtype are shown. The scale represents number of base substitutions per site.

(C)

FIGURE 4.1 *(continued)*

TABLE 4.2

Minor Mutations and Natural Polymorphisms Detected in the Protease of Drug-naive Patients from Mozambique

Resistance mutations and polymorphisms[a]	Patients (%)						P value (Mozambique vs World) [b]			
	Mozambique			World[a]						
	C (n = 86)	Non-C (n = 13)	*P*[b]	C (n = 1684)	B (n = 6059)	*P*[b]	C_{MZ} vs C_W	C_{MZ} vs B_W	Non-C_{MZ} vs C_W	Non-C_{MZ} vs B_W
L10V	0	8	0.1313	1	3	<0.0001	1	0.1898	0.1299	0.8605
V11I	0	54	<0.0001	0	0	na	na	na	<0.0001	<0.0001
T12S	78	8	<0.0001	64	4	<0.0001	0.0078	<0.0001	<0.0001	0.9771
I15V	84	15	<0.0001	85	15	<0.0001	0.7572	<0.0001	<0.0001	0.7262
L19I	74	8	<0.0001	59	6	<0.0001	0.0046	<0.0001	0.0002	0.7423
K20I	0	77	<0.0001	0	0	na	na	na	<0.0001	<0.0001
M36I	94	100	1	83	13	<0.0001	0.0042	<0.0001	0.1426	<0.0001
R41K	85	100	0.2065	77	23	<0.0001	0.1116	<0.0001	0.0485	<0.0001
Q58E	1	0	1	0	0	na	0.0486	<0.0001	na	na
L63P	30	15	0.3400	30	56	<0.0001	1	<0.0001	0.3662	0.0077
H69K	99	100	1	99	2	<0.0001	0.5938	<0.0001	1	<0.0001
L89M	79	100	0.1175	85	2	<0.0001	0.1650	<0.0001	0.2368	<0.0001
I93L	95	8	<0.0001	94	29	<0.0001	0.8150	<0.0001	<0.0001	0.1658

Minor mutations are in bold letters.

Na, not applicable; [a], according to the Stanford HIV Drug Resistance Database; [b], Fisher exact test.

TABLE 4.3

Demographic, Immunologic and Virologic Characteristics of the Patients Harboring Transmitted Drug Resistance Mutations

Patient	Sampling year	Gender	Age	Number of CD4 cells/µl	HIV-1 RNA (log copies /ml)	Genetic form	
						PR	RT
04MZUNIV87	2002	F	26	382	4.57	C	U
04MZUNIV15	2003	M	39	224	5.35	C	U
04MZUNIV12	2003	M	41	33	5.76	C	C
04MZUNIV122	2003	M	38	301	5.23	U	C

Resistance Mutations			Predicted susceptibility to RT inhibitors								
PI	NRTI	NNRTI	3TC	ABC	AZT	D4T	DDI	FTC	TDF	EFV	NVP
n	M41L, D67N, M184V, L210W, T215Y	n	R	R	R	R	I	R	I	S	S
n	M41L, M184V, T215F	K103N	R	I	I	I	I	R	PLLR	R	R
n	M41L, M184V, T215F	n	R	I	I	I	I	R	PLLR	S	S
n	K219Q	n	S	S	LLR	PLLR	S	S	S	S	S

3TC, lamivudine; ABC, abacavir; AZT, zidovudine; D4T, stavudine; DDI, didanosine; FTC, emtricitabine; TDF, tenofovir; EFV, efavirenz; NVP- nevirapine; R, high-level resistance; S, susceptible; I, intermediate resistance; LLR, low-level resistance; PLLR, potential low-level resistance; n, no resistance mutations; U, untypable.

Discussion

The primary goals of this study were to determine, for the first time, the prevalence of transmitted HIV-1 drug resistance in antiretroviral-naive patients attending the main public hospitals and private clinics in Maputo in 2002-2004 and to identify the HIV-1 genetic forms present in this population. Maputo is the capital city of Mozambique and has the third highest HIV-1 prevalence rates in the country (23%, 18-29%) [588].

Unlike other studies investigating transmitted HIV drug resistance in Mozambique [147, 599, 600] and elsewhere in Africa [254, 257-259, 584], the patients enrolled in our study could afford to pay for CD4 cell counts, plasma viral load and, eventually, for antiretroviral drugs. Moreover, they were, in general, severely immune compromised. In fact, based on the WHO recommendations for ART eligibility in adults from resource-constrained settings (CD4 cell count below 200 cells/µl), 38% of our patients qualified for ART [605]. Low CD4 counts were directly related with high viral load which is consistent with the reported absence of ART [606]. Notably, however, viral load was undetectable in 12 (8%) patients. When compared to the patients with detectable plasma viremia, these patients had a significantly higher number (and percentage) of CD4 cells and lower percentage of CD8 cells. These patients may be HIV-1 elite controllers [604].

We obtained a 61% genotyping success rate using an in-house method and samples that were inadequately conserved most of the time (-20 ^{o}C). Most patients with amplifiable virus were subtype C. This success rate compares favourably to that obtained in recent studies performed in South Africa (51%, samples collected in 2002; 41%, 2004) [258] and Ethiopia (52%, 2005) [254], where subtype C prevails. The amplification and sequencing primers that we use perform well with the most divergent HIV-1 isolates [167, 570] and this, given the high genetic diversity of HIV in Mozambique (see below), may have been crucial for our relatively high genotyping success rate.

Most of our patients (80.8%) were infected with subtype a C virus which, together with previous studies, demonstrates that this is the prevailing subtype in the Center and South of the country [147, 599, 600]. Phylogenetic analysis revealed that the C viruses circulating in Maputo are highly divergent and probably have been introduced into this city from diverse geographic regions. These results are consistent with previous findings demonstrating the existence of many subtype C sub-lineages in southern Africa [607]. Unlike all other studies performed in Mozambique, we could detect in an important proportion (19.2%) of patients all other major African subtypes and untypable variants, either alone (subtype G) or in recombinant forms with subtype C (A, D, F and U) [147, 599, 600]. Notably, phylogenetic analysis has shown that a cluster of 7 patients was infected with a highly homologous strain that is closely related to CRF37_cpx, a recombinant originally found in Cameroon [60], and to sub-subtype A3, originally found in Senegal [608]. This type of clustering usually results from a founder effect and strongly suggests that these strains were recently introduced into Maputo [180].

TABLE 4.4

Resistance Mutations and Natural Polymorphisms Detected in the Reverse Transcriptase of Drug-naive Patients from Mozambique

Resistance mutations and polymorphisms[a]	Patients (%)						*P* (Mozambique vs World)[b]			
	Mozambique			World[a]						
	C (n = 59)	Non-C (n = 9)	*P*[b]	C (n = 1950)	B (n = 4545)	*P*[b]	C_{MZ} vs C_W	C_{MZ} vs B_W	Non-C_{MZ} vs C_W	Non-C_{MZ} vs B_W
V35T	80	77	1	93	0	<0.0001	0.0003	<0.0001	0.1281	<0.0001
E36A	68	11	0.002	77	0	<0.0001	0.1344	<0.0001	<0.0001	<0.0001
T39E	79	0	<0.0001	68	0	<0.0001	0.0792	<0.0001	<0.0001	na
M41L	2	22	0.0441	0	0	na	0.0053	<0.0001	<0.0001	<0.0001
S48T	58	11	0.012	87	2	<0.0001	<0.0001	<0.0001	<0.0001	0.4505
D67N	0	11	0.1324	0	0	na	na	na	0.0046	<0.0001
K103N	0	11	0.1324	0	0	na	na	na	0.0046	<0.0001
V118I	5	0	1	2	2	0.9276	0.2421	0.2301	1	0.4452
K122E	89	44	0.004	90	27	<0.0001	0.8592	<0.0001	0.0009	0.4227
D177E	58	89	0.138	76	21	<0.0001	0.0021	<0.0001	0.6955	<0.0001
M184V	2	22	0.0441	0	0	na	0.0053	<0.0001	<0.0001	<0.0001
T200A	91	78	0.230	89	16	<0.0001	0.6928	<0.0001	0.2607	<0.0001
Q207E	70	67	1	64	12	<0.0001	0.4661	<0.0001	1	<0.0001
L210W	2	22	0.0441	0	0	na	0.0053	<0.0001	<0.0001	<0.0001
R211K	77	100	0.187	62	42	<0.0001	0.0363	<0.0001	0.0162	0.0015
T215F	2	11	0.2489	0	0	na	0.0053	<0.0001	0.0046	<0.0001
T215Y	0	11	0.1324	0	0	na	na	na	0.0046	<0.0001

TABLE 4.4 *(continued)*

Resistance mutations and polymorphisms[a]	Patients (%)						*P* (Mozambique vs World)[b]			
	Mozambique			World[a]						
	C (n = 59)	Non-C (n = 9)	*P*[b]	C (n = 1950)	B (n = 4545)	*P*[b]	C_{MZ} vs C_W	C_{MZ} vs B_W	Non-C_{MZ} vs C_W	Non-C_{MZ} vs B_W
K219Q	2	0	1	0	0	na	0.0053	<0.0001	na	na
V245Q	61	67	1	83	2	<0.0001	<0.0001	<0.0001	0.1869	<0.0001
A272P	86	33	0.002	80	50	<0.0001	0.2905	<0.0001	0.0032	0.5054
T286A	70	56	0.456	73	26	<0.0001	0.6504	<0.0001	0.2638	0.1016
E291D	84	56	0.060	95	2	<0.0001	0.0015	<0.0001	0.0007	<0.0001
V292I	84	44	0.015	94	8	<0.0001	0.0089	<0.0001	<0.0001	0.0007
I293V	95	78	0.127	95	46	<0.0001	0.8547	<0.0001	0.0835	0.1151

Resistance mutations are in bold letters.

na, not aplicable; [a], according to the Stanford HIV Drug Resistance Database; [b], Fisher exact test.

In summary, our results indicate that the HIV-1 epidemic in Maputo is evolving rapidly to a greater epidemiological and virological complexity when compared to the rest of the country where subtype C prevails [147, 599, 600]. It will be important to monitor the potential impact of the multiple HIV-1 genetic forms (and HIV-2) in ART, especially now that it is being universally expanded, in diagnosis and in vaccination.

In contrast with a previous study performed in Beira [600] we did not find major PI resistance mutations. The Q58E minor mutation, which has been associated with decreased virological response to tipranavir [576], was detected in only 1 subtype C isolate. Interestingly, however, V11I was detected in all 7 CRF37_cpx infected drug-naive patients. We found it also in the CRF37_cpx reference sequence from Cameroon [60]. V11I is one of the 11 mutations associated with resistance to darunavir [485, 486]. It occurs in only 1.8% and 1.3% of CRF02_AG and subtype G isolates from drug-naive patients, respectively, but is even more rare (<1%) in the remaining subtypes [574]. Therefore, our results indicate that V11I is a natural polymorphism of CRF37_cpx isolates, and suggest that these viruses may have a lower genetic barrier to darunavir resistance.

We could detect NRTI-and/or NNRTI-resistant variants in 4 (5.9%) patients enrolled in 2002 (1 patient) and 2003 (3 patients). These patients were infected with subtype C/C (1 patient), U/C (1 patient) and C/U (2 patients) recombinant strains. Patients with drug resistant virus had a significantly lower viral load compared to rest of the patients suggesting a lower replicative capacity of the transmitted resistant variants [609]. Resistance to the NRTIs was due to the most frequent thymidine analogue-associated mutations (M41L, D67N, L210W, T215F, T215Y, K219Q) and M184V [574, 575]. One (1.5%) isolate was also resistant to the NNRTIs due to K103N. These results predict that none of these isolates would be fully sensitive to the standard first-line antiretroviral regimens used in most resource limited settings, including Mozambique [580, 609]. Interestingly, phylogenetic analysis revealed that 2 C/U recombinant isolates (04MZUNIV87 and 04MZUNIV15) clustered together in the RT region which could be an indication of epidemiological linkage between the 2 patients. These 2 isolates, however, had different resistance mutations and profiles (1 was fully resistant to the NNRTIs and 2 NRTIs whereas the other was resistant to 5 NRTIs) and their PR region was phylogenetically unrelated. Therefore, the results suggest that 2 drug resistant U isolates were imported into Maputo, possibly from the same geographic region, and recombined locally with different C strains.

The finding of transmitted HIV-1 drug resistance in Maputo was unexpected in view of the relatively restricted availability of antiretroviral drugs until 2004 [598]. In fact, 2 other studies performed in urban [599] and rural [147] villages located near Maputo did not find evidence for transmission of drug resistant HIV-1. Transmission of resistant HIV-1 variants has, however, been previously detected in Beira in 2003 in 9.3% of patients [600]. The 2 most likely explanations for the origin of the resistant isolates in the 2 major cities of Mozambique are as follows: 1) The unregulated and unmonitored use of antiretroviral drugs [585]; 2) People displacements from countries where ART is available for a longer period [583].

This study has 2 main limitations that may have biased in opposite ways the estimated number of transmitted HIV-1 drug resistance in Maputo. First, the demographic, virologic and immunologic characteristics of our patients suggest that most of them were not recently infected [582]. Moreover, these patients had a relatively high socio-economic status since they could afford to pay for lab analysis. Therefore, the likelihood that they have had previous unreported contact with antiretroviral drugs is high and we may be over estimating the number of transmitted HIV drug resistant strains [582]. Second, we have used a bulk sequencing strategy in inadequately conserved samples both of which severely compromise detection of HIV-1 minority-resistant variants [609].

Our results are particularly important in view of the recent increase in ART programs in Mozambique which brings free-of-charge, standard first-line antiretroviral regimens to patients requiring treatment for their advanced disease and to pregnant women to prevent mother-to-child transmission [582, 599, 610, 611]. Overall, the low prevalence of transmitted HIV drug resistance is reassuring and indicates that these simplified first-line regimens can be successfully used in the vast majority of HIV-1 patients from Maputo. Continued surveillance of drug resistance in treated and untreated populations is important to maximize the efficacy of ART in Maputo.

CHAPTER 5

Genetic diversity and drug resistance profiles in HIV-1 and HIV-2 infected patients from Cape Verde Islands

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Abstract

Our aim was to characterize for the first time the genetic diversity of HIV in Cape Verde Islands as well as the drug resistance profiles in treated and untreated patients. Blood specimens were collected from 41 HIV-1 and 14 HIV-2 patients living in Santiago Island. Half of the patients were on ART. *Pol* and *env* gene sequences were obtained using in-house methods. Phylogenetic analysis was used for viral subtyping and the Stanford Algorithm was used for resistance genotyping. For HIV-1, the amplification of *pol* and *env* was possible in 27 patients (66%). HIV-1 patients were infected with subtypes G (13, 48%), B (2, 7%), F1 (2, 7%) and CRF02_AG (2, 7%), and complex recombinant forms including a new C/G variant (n=8, 30%). Drug resistance mutations were detected in the PR and RT of 3 (10%) treated patients. M41L and K103N transmitted drug resistance mutations were found in 2 of 17 (12%) untreated patients. All 14 HIV-2 isolates belonged to group A. The origin of 12 HIV-2 strains was impossible to determine whereas 2 strains were closely related with the historic ROD strain. In conclusion, in Cape Verde there is a long-standing HIV-2 epidemic rooted in ROD-like strains and a more recent epidemic of unknown origin. The HIV-1 epidemic is caused by multiple subtypes and complex recombinant forms. Drug resistance HIV-1 strains are present at moderate levels in both treated and untreated patients. Close surveillance in these two populations is crucial to prevent further transmission of drug resistant strains.

Introduction

Cape Verde is an archipelago located offshore Senegal in West African coast with a population of 491,575 individuals [612]. The first AIDS case in a Cape Verdian individual was identified in 1982 and was caused by HIV2 [6]. Similarly to neighbouring countries such as Senegal and Gambia, HIV seroprevalence is low, estimated at less than 1% according to the national surveys from 1989 and 2005 [613]. At the end of 2005, approximately 1,710 cases of HIV infection were notified, and half were already in AIDS stage. At the end of 2005, approximately 1,710 cases of HIV infection were notified, and half were already in AIDS stage. Similarly to other West African countries, both types of HIV circulate in the country with 72% of the notified cases being caused by HIV-1, 22% by HIV-2 and 6% by both viruses (potential double infections) [613].

Since the advent of highly active antiretroviral therapy in developed countries we have seen an improved prognosis of HIV-1 infected patients, with a significant decrease in morbidity and mortality rates associated with this infection [13, 14]. Combined antiretroviral therapy (cART) has been introduced recently in resource-limited countries [614]. However, there are concerns on the low efficacy of cART in these countries which could lead to the rapid and uncontrolled emergence and transmission of drug resistant viruses [615]. Three aspects that can lead to low cART efficacy are the limited HIV-RNA monitoring of patients undergoing therapy, the unregulated and unmonitored use of antiretroviral drugs bought in the black market or abroad, and the lack of drug resistance surveillance systems [585, 615-617]. Despite these limitations, transmitted HIV drug resistance is still residual (<5%) in most Sub-Saharan countries [148, 254-259].

Worldwide strains of HIV-1 are phylogenetically classified into four very divergent groups, M, N, O and P [32]. Amino acid sequences of Gag and Env can differ between groups up to 30% and 47%, respectively. HIV-1 group M, the only pandemic group, has diversified into nine divergent subtypes (A, B, C, D, F, G, H, K and J), six sub-subtypes (F1, F2, A1-A5) and multiple circulating recombinant forms [51, 105]. Amino acid sequences of Env can differ up to 20% within a particular subtype and over 35% between subtypes. As for HIV-2, which is mostly restricted to a few West African countries including Cape Verde, despite the existence of 8 genetic groups named A to H only groups A and B seem to be spreading, with group A much more common than group B [70-72].

The extraordinary HIV diversity is seen as a major hurdle to the development of a prophylactic vaccine [398]. HIV diversity and its rapid evolution may present significant problems to diagnosis and antiviral therapy. For example, significant antigenic changes in HIV-1 group O viruses have recently been shown to hinder the diagnostic performance of several serodiagnostic assays [311]. Likewise, many comparative studies have shown that the sensitivity and specificity of viral load assays varies depending on HIV-1 group or subtype [32, 187, 618-620]. Regarding antiviral therapy, there are major differences in the susceptibility of HIV-1 and HIV-2 to the current available drugs. Indeed, HIV-2 is naturally resistant to non nucleoside reverse transcriptase inhibitors (NNRTIs) and it presents a diminished sensitivity to certain protease inhibitors (PI) [621]. Moreover, most cART regimens used in HIV-1 patients are unable to fully suppress HIV-2 replication, to increase the

number of CD4 cells or prevent accumulation of drug resistant mutations [622]. Diversity among HIV-1 isolates also affects antiviral therapy. For instance, like HIV-2, group O strains are resistant to NNRTIs [432, 623]. Natural polymorphisms found in the protease and reverse transcriptase of some non-B subtypes may lower their genetic barrier to resistance to some protease and reverse transcriptase inhibitors [256, 624]. Finally, certain drug resistant mutations accumulate more frequently and rapidly in some non-B subtypes than in HIV-1 B (e.g. K65R, L90M and V106M in subtype C and I54V/L in subtype G) [490, 495, 624]. Hence, HIV diversity has major implications for the development of resistance, for genotypic evaluation of drug resistance and for the selection of therapeutic regimens, at least in countries where both HIV-1 and HIV-2 circulate such as Cape Verde.

Cape Verde, like most other resource-limited countries, adopted the World Health Organization (WHO) public health approach in antiretroviral therapy (ART) utilization [580]. cART is available free of charge since December of 2004 and was initially given to a group of 148 patients. The preferential first line regime adopted includes a combination of lamivudine (3TC), stavudine (d4T) and nevirapine (NVP). Presently, there is no data about the genetic diversity of HIV in Cape Verde. Moreover, there is no information about the prevalence and nature of primary and secondary drug resistance in this country. The aim of the present work was to determine, for the first time, the HIV-1 and HIV-2 genetic forms present in Cape Verde and the drug resistance profiles in treated and untreated patients.

Materials and Methods

Patients

Blood samples were collected during 2005 (n=37), 2006 (n=14) and 2007 (n=4) from 41 HIV-1 and 14 HIV-2 infected patients living in the Santiago Island and attending the Delegacia de Saúde, Cidade da Praia, Cape Verde. Nineteen of the HIV-1 individuals had received cART, 18 were drug-naive and for 4 there were no indication on the drug regimen. Half of the HIV-2 patients were on cART. The epidemiological characteristics of the HIV-1 and HIV-2 patients are described in Table 5.1. Plasma samples were preserved at -20 ^{o}C in Cidade da Praia and thereafter at -80 ^{o}C in Lisbon, Portugal. Serologic diagnosis of HIV infection and CD4+ T cell counts were done in Cape Verde; plasma viral load was determined with the Cobas Amplicor HIV-1 Monitor test version 1.5 (Roche) in Lisbon, Portugal. Written informed consent was obtained from all participants and the study was approved by the ethics committees of the participating institutions.

Viral RNA Extraction, PCR Amplification and Sequencing

HIV-1 viral RNA was extracted from 140 µl of plasma using QIAamp Viral RNA miniKit (QIAGEN). Reverse transcriptase–polymerase chain reaction was performed with Titan One Tube reverse

transcriptase–polymerase chain reaction System (Roche). Nested polymerase chain reaction was done to obtain a 409 bp fragment from the C2V3C3 *env* region, a 532 bp fragment containing the part of the *gag* gene and all the PR region and a 1026 bp fragment from the RT region. Thermal cycling conditions and primers sequence and position were described elsewhere [167, 625]. HIV-2 viral RNA was extracted from plasma as described above. Nested polymerase chain reaction was done to obtain a 373 bp fragment from the C2V3C3 *env* region as described elsewhere [626]. DNA sequences were obtained with Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems) and an automated sequencer.

Table 5.1

Comparison of Demographic, Immunologic and Virologic Characteristics Between HIV-1 and HIV-2 Infected Patients

Variables	HIV-1	HIV-2	P value
N (%)	41 (75)	14 (25)	-
Gender N (%)			
Male	12 (29)	6 (43)	0.5253[a]
Female	25 (61)	8 (57)	
Unknown	4 (10)		-
Age group (years) N (%)			
10-19	1 (3)	0	
20-29	3 (7)	1 (7)	
30-39	12 (29)	3 (21)	0.5217[b]
40-49	14 (34)	6 (43)	
50-59	7 (17)	4 (29)	
Unknown	4 (10)	-	-
Transmition route			
Sexual	33 (81)	13 (93)	
Sexual/Blood transfusion	1 (2)	1 (7)	1.000[c]
Sexual/Intravenous drug user	2 (5)	-	
Vertical	1 (2)	-	
Unknown	4 (10)	-	-
CD4 (median, range), cells/µl	417 (14-820)	487 (35-1420)	0.4115[d]
HIV RNA (mean, SD), copies/ml (N= 23)	9.4×10^4 (1.3×10^5)	nd	-
ART (N, %)	19 (46)	7 (50)	1.000[c]

ART - antiretroviral therapy; nd - not done; [a]Fisher exact test; [b]Fisher exact test comparing the number patients below or above 40 years old; [c]Fisher exact test comparing sexual transmission with other routes of transmission; [d]Mann-Whitney test; [e]Fisher exact test comparing the number patients on ART with untreated patients.

Phylogenetic and Recombination Analysis

Sequences were aligned with sequences from reference strains collected from the Los Alamos Sequence Database (http://hiv.lanl.gov) using ClustalX 1.8 [195]. Maximum likelihood phylogenetic analyses [551] were performed using the best-fit model of molecular evolution estimated by Modeltest v3.7 under the Akaike Information Criterion [552]. The chosen models were K81uf+I+G for the *env* gene and GTR+I+G for the *pol* gene of HIV-1 sequences and the TrN+I+G for both PR and C2V3C3 HIV-2 regions. Tree searches were conducted in PAUP v4.0b10 using a nearest neighbor interchange heuristic search strategy [627] and bootstrap resampling [628]. Bootstrap values ≥ 70% were considered definitive for significant clustering [629]. All sequences were checked for hypermutation at the Los Alamos website using the program Hypermut [630].

Resistance Mutation Analysis

Genotypic analysis of HIV-1 drug resistance was performed using the Stanford Genotypic Resistance Interpretation Algorithm (http://hivdb.stanford.edu/index.html). At first, we considered all resistance mutations and unusual polymorphisms. Mutations associated with transmitted HIV drug resistance were then selected from a recently published list [249].

Statistical Analysis

Mann–Whitney test was used to compare groups. The frequencies of drug resistance mutations of viruses from Cape Verde were compared with those of all subtype B and G sequences available at the Stanford HIV Drug Resistance Database using Fisher exact test. P values <0.05 were considered significant.

Results

We analyzed samples from 55 patients, 41 (75%) infected with HIV-1 and 14 (25%) infected with HIV-2. There were no significant epidemiological or immunological differences between HIV-1 and HIV-2 infected patients (Table 5.1). Most patients (60%) were female and were in the age group of 40-49 years. The infection was mostly transmitted by the sexual route. Most patients had less than 500 CD4+ T cells which is suggestive of long term infection. As expected, viral load was significantly lower in treated HIV-1 patients (N=18) than in untreated patients (N=19) (median HIV RNA copies per ml of plasma; range, 400; 400-93,000 *vs* 43900; 400-513,000; P < 0.0001).

For HIV-1, the amplification of the PR, RT and/or C2V3C3 genomic regions was possible in 27 HIV-1 samples (66%). Ten of these patients (37%) were on cART and 17 (63%) were drug-naive individuals. PR and RT sequences were obtained in 26 (63%) and 27 (66%) patients, respectively. C2V3C3 *env* gene sequences were obtained in 24 (59%) patients.

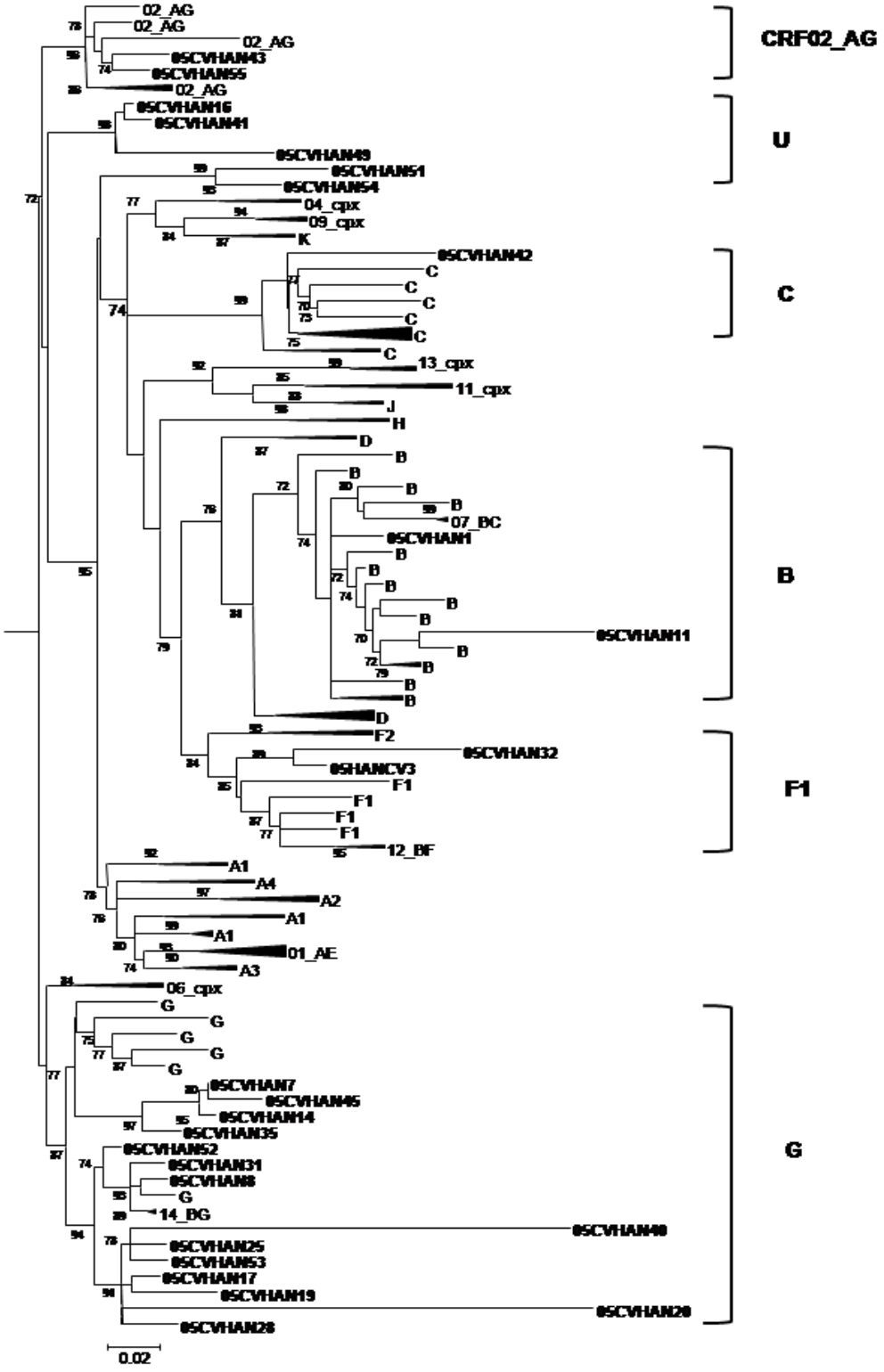
Phylogenetic analyses revealed that 13 (48%) viral isolates were subtype G, six (22%) were non-G [two (7%) subtype B, two (7%) subtype F1 and two (7%) CRF02_AG], four (15%) were recombinants between subtype G and other subtypes or U strains [1 G (PR)/ G (RT)/ H (C2V3C3); 1 G/G/B; 1 C/C/G; 1 U/G/-], two (7%) were recombinants between CRF02_AG and subtype B and/or U strains [1 U/B/CRF02_AG; 1 U/CRF02_AG/CRF02_AG] and two (7%) were recombinants between A subtype and U strains (U/A/U) (Figure 5.1).

There were no *major* mutations associated with PI resistance in the naive population. The *minor* mutations associated with PI resistance, L10I (6%) and A71V (6%), were detected in two unrelated drug-naive patients (Table 5.2). The M41L, K103N and Y318F mutations conferring resistance to NRTIs or NNRTIs were found in 3 of 17 untreated patients. Only M41L and K103N are considered transmitted drug resistance mutations [249, 575, 631]. Hence, our results reveal a 12% (2 out of 17 patients) rate of transmitted drug resistance in Cape Verde.

Resistance mutations were detected in 3 of 10 (30%) patients undergoing cART (Table 5.3). No *major* mutations associated with PI resistance were found in this population. The *minor* mutations L10I/V and V11I, associated with PI resistance, were found in two patients infected with complex recombinant isolates. The K65R, T69d, K103N and Y181C mutations, which confer resistance to the NRTIs and NNRTIs, were all detected in patient 05HANCV55 which was infected with a CRF02_AG strain.

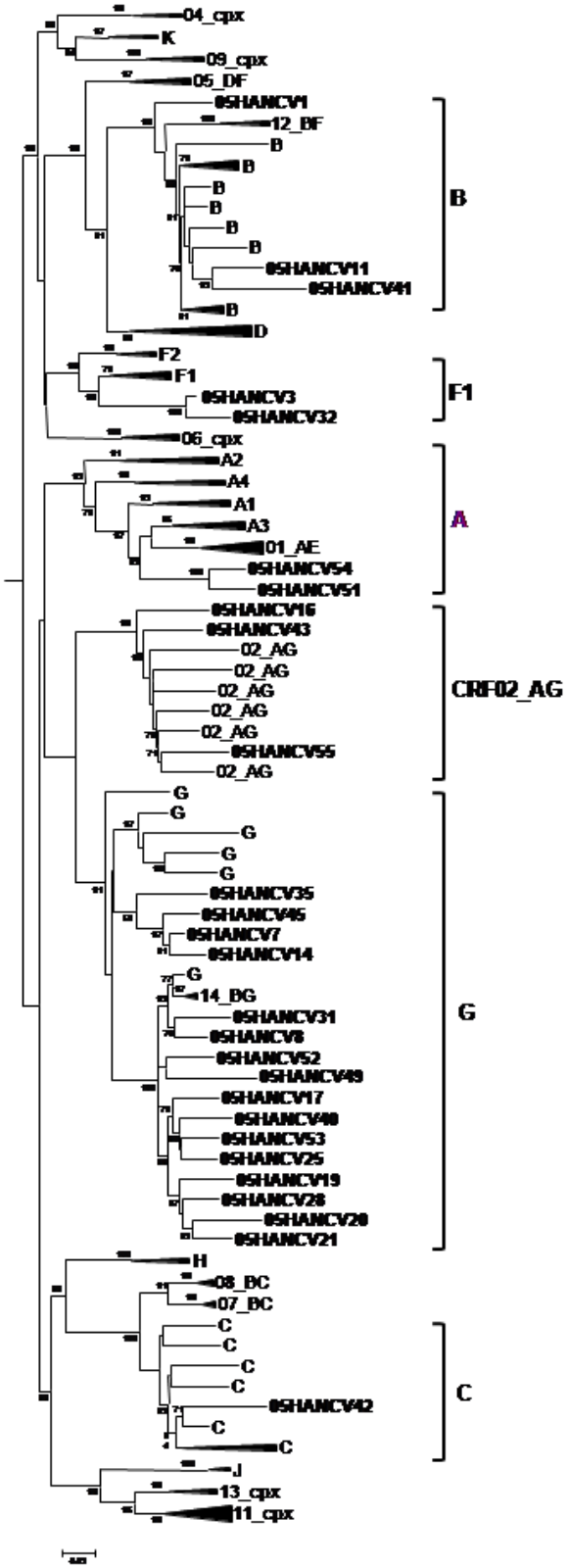
The frequency of some previously known PR polymorphisms detected in subtype G isolates (29% of polymorphisms) was significantly different ($P < 0.05$) when compared to isolates of similar subtype found throughout the world (Figure 5.2). Likewise, the frequency of the RT polymorphisms found in G subtype (38% of polymorphisms) was significantly different ($P <0.05$) when compared to isolates from worldwide treatment-naive patients infected with the same subtype (Figure 5.3). These results are a clear indication that the G strains circulating in Cape Verde are highly divergent and genetically complex. The origin of the G strains from Cape Verde was investigated by phylogenetic analysis using RT and C2V3C3 *env* sequences from reference G sequences collected worldwide as well as sequences from Angola and Portugal deposited in the Los Alamos Database. We found that the majority of the sequences from Cape Verde were closely related with sequences from Portugal and/or Angola (100% in the RT and 91% in *env*) (Figure 5.4). These results indicate that the HIV-1 variants circulating in Cape Verde have their origin in one of these two countries.

All HIV-2 isolates were classified as group A (Figure 5.5). Eleven out of 14 isolates (76%) formed two clusters of unknown origin. Remarkably, however, isolates 05HANCV26 and 05HANCV36 revealed a strong evolutionary relationship with reference sequences from Guinea-Bissau and with the historic isolate HIV-2ROD. These results demonstrate that ROD-like strains are still circulating in this country (Figure 5.5).



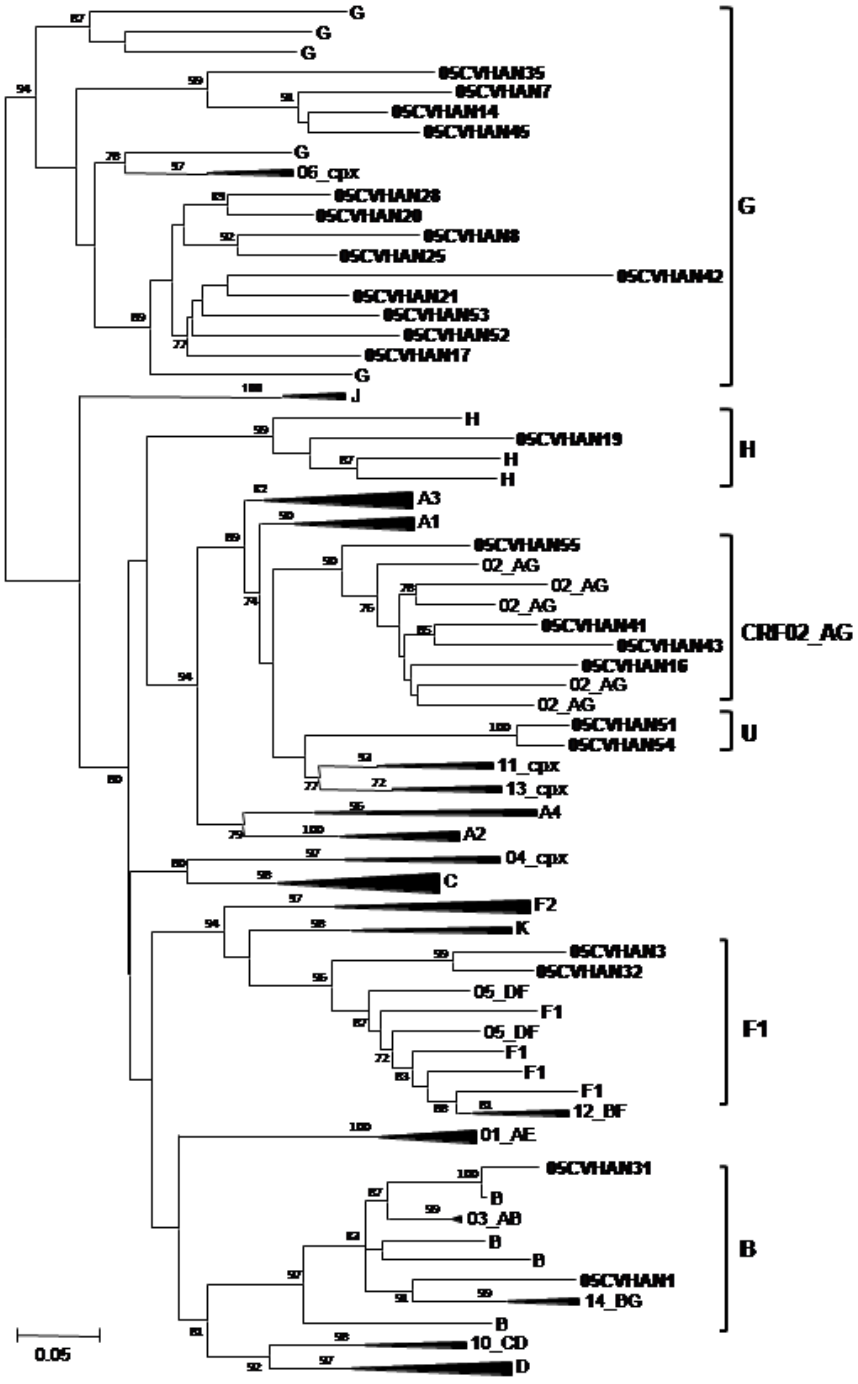
(A)

FIGURE 5.1 - Genetic subtypes and evolutionary relationships of the HIV-1 viruses sequenced in this study based on maximum likelihood phylogenetic trees of PR (A), RT (B) and C2V3C3 *env* gene region (C). The phylogenetic trees were constructed with reference sequences from all HIV-1 genetic forms obtained from different locations. Sequences from Cape Verde isolates are shown in bold letters.



(B)

FIGURE 5.1 (*continued*)



(C)

FIGURE 5.1 (*continued*)

TABLE 5.2

Resistance Mutations Detected in Drug-naive Patients and Predicted Susceptibility to Antiretroviral Drugs

Patient	Genetic form			Resistance Mutations			Predicted susceptibility to RT inhibitors									
	PR	RT	*env*	PI	NRTI	NNRTI	3TC	ABC	AZT	D4T	DDI	FTC	TDF	EFV	ETR	NVP
05HANCV8	G	G	G			K103N	S	S	S	S	S	S	S	R	S	R
05HANCV11	B	B	-	L10I												
05HANCV20	G	G	G	A71V												
05HANCV31	G	G	B		M41L		S	PLLR	LLR	LLR	PLLR	S	PLLR	S	S	S
05HANCV45	G	G	G			Y318F	S	S	S	S	S	S	S	PLLR	PLLR	I

3TC, lamivudine; ABC, abacavir; AZT, zidovudine; D4T, stavudine; DDI, didanosine; FTC, emtricitabine; TDF, tenofovir; EFV, efavirenz; ETR, etravirine; NVP, nevirapine; R, high-level resistance; S, susceptible; I, intermediate resistance; LLR, low-level resistance; PLLR, potential low-level resistance; n, no resistance mutations; U, untypable. PLLR, potential low-level resistance; n, no resistance mutations; U, untypable.

TABLE 5.3

Drug Resistance Mutations Detected in Patients Undergoing ART from Cape Verde

Patient	ART regimen	HIV-1 RNA (copies /ml)	Genetic form		
			PR	RT	*env*
05HANCV16	AZT+3TC+NVP	1690	U	02_AG	02_AG
05HANCV41	3TC+d4T+NVP	<400	U	B	02_AG
05HANCV55	Unknown	93000	02_AG	02_AG	02_AG

Resistance Mutations			Predicted susceptibility to RT inhibitors									
PI	NRTI	NNRTI	3TC	ABC	AZT	D4T	DDI	FTC	TDF	EFV	ETR	NVP
L10I, V11I	-	-	-	-	-	-	-	-	-	-	-	-
V11I	-	-	-	-	-	-	-	-	-	-	-	-
L10V	K65R	-	I	I	S	LLR	I	I	I	S	S	S
-	T69d	-	LLR	LLR	I	I	LLR	LLR	LLR	S	S	S
-	-	Y181C	S	S	S	S	S	S	S	I	I	R
-	-	K103N	S	S	S	S	S	S	S	R	S	R

3TC, lamivudine; ABC, abacavir; AZT, zidovudine; D4T, stavudine; DDI, didanosine; FTC, emtricitabine; TDF, tenofovir; EFV, efavirenz; ETR, etravirine; NVP, nevirapine; R, high-level resistance; S, susceptible; I, intermediate resistance; LLR, low-level resistance; PLLR, potential low-level resistance; n, no resistance mutations; U, untypable.

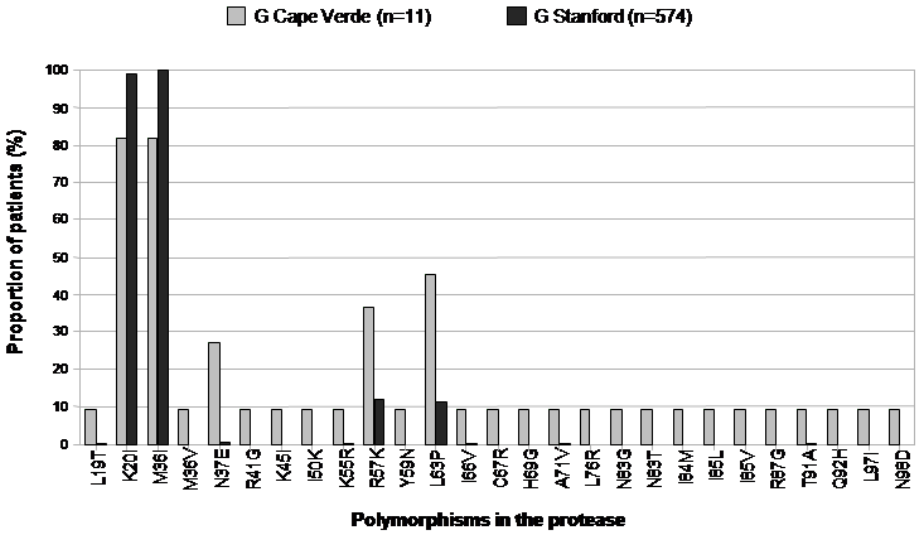


FIGURE 5.2 - Minor mutations and natural polymorphisms detected in the protease of drug-naive patients from Cape Verde. Only the mutations whose frequency is significantly different (P < 0.05, Fisher's exact test) than that found in the Stanford database for untreated patients are shown.

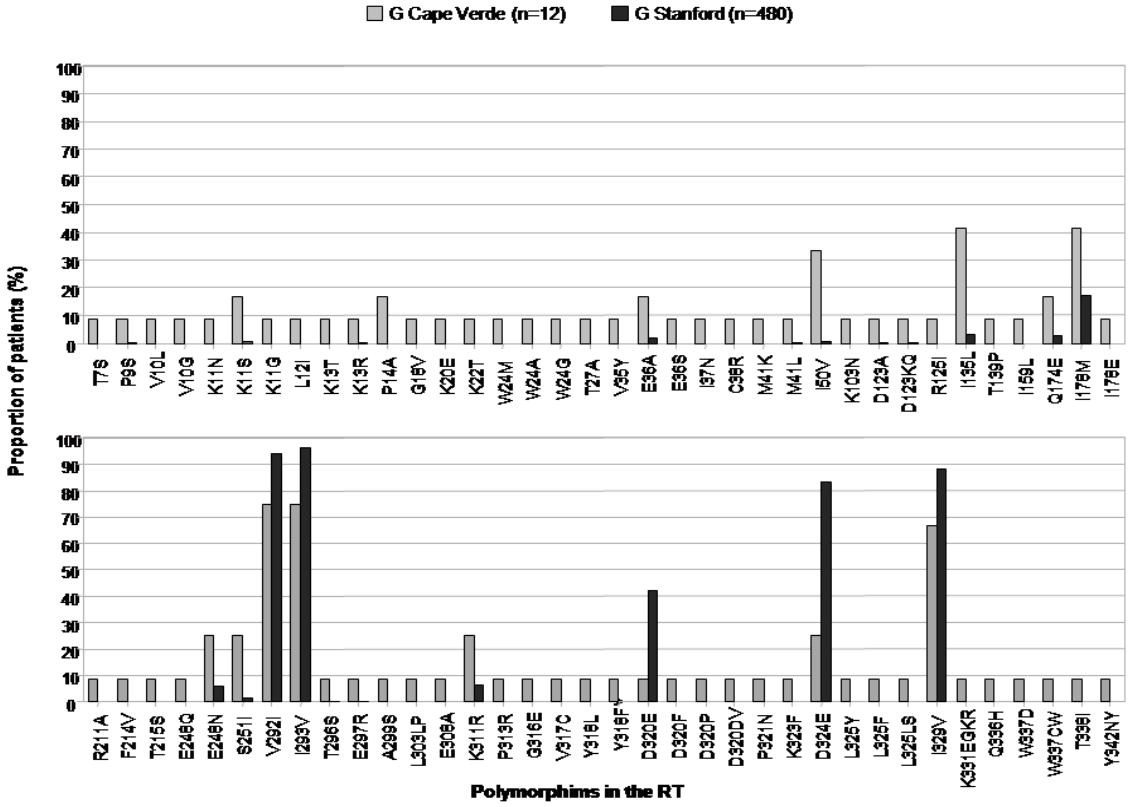


FIGURE 5.3 - Resistance mutations and natural polymorphisms detected in the RT of drug-naive patients from Cape Verde. Only the mutations whose frequency is significantly different (P < 0.05, Fisher's exact test) than that found in the Stanford database for untreated patients are shown.

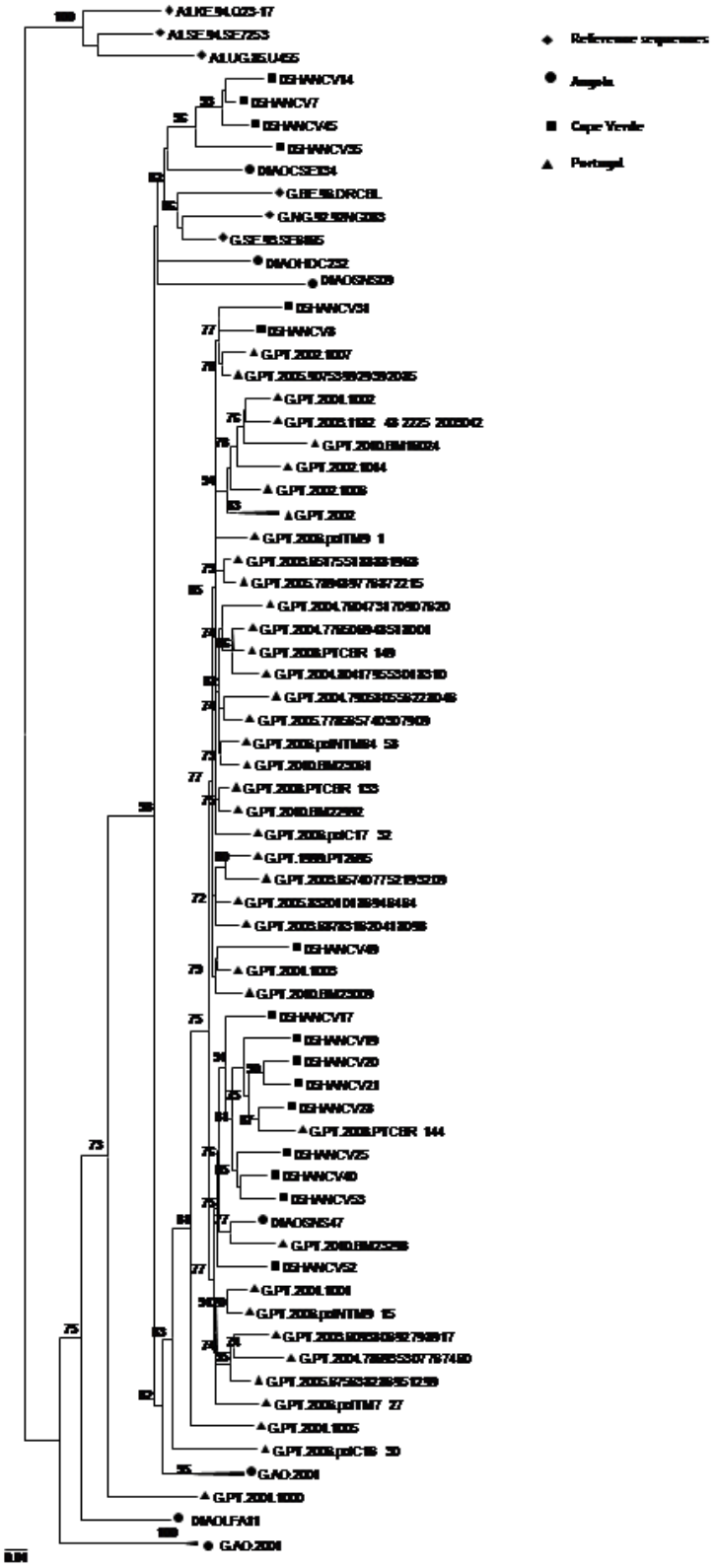


FIGURE 5.4 – Origin of the HIV-1 subtype G isolates from Cape Verde. Phylogenetic trees were built using RT (A) and C2V3C3 (B) genomic regions of the G isolates from this study as well as G sequences from reference isolates and from Angolan (AO) and Portuguese (PT) patients, all downloaded from the Los Alamos database. Trees were built as described in the methods section. Bootstrap values of 70% or greater indicate significant support for the clusters. The scale represents number of base substitutions per site.

(A)

(B)

FIGURE 5.4 *(continued)*

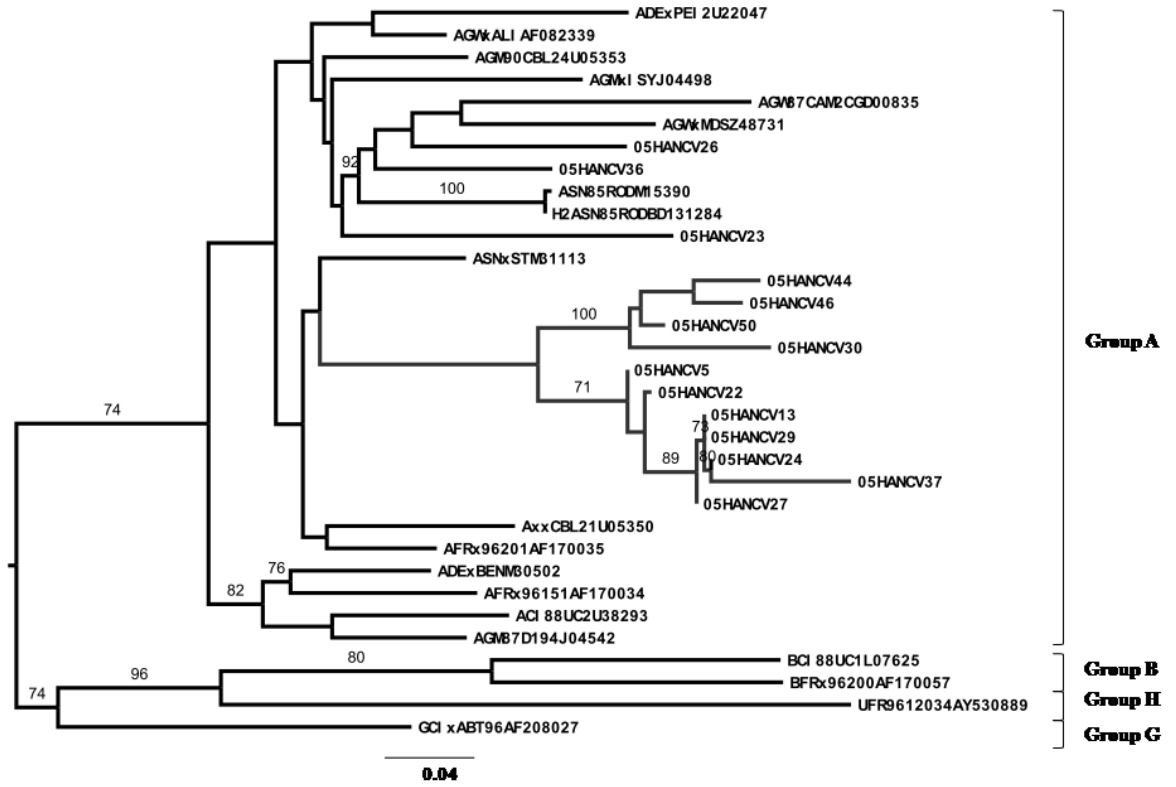


FIGURE 5.5 - Genetic groups and evolutionary relationships of the HIV-2 viruses sequenced in this study based on maximum likelihood phylogenetic trees of C2V3C3 *env* gene region. The phylogenetic tree was constructed with reference sequences from all HIV-2 groups. Sequence from Cape Verde isolates start with 05HANCV.

Discussion

This work is the first characterization of the HIV-1 and HIV-2 genetic forms present in Cape Verde Islands and the drug resistance profiles in treated and untreated patients. HIV-1 G was the prevailing subtype in our patients (48%) but most other subtypes and some untypable variants were also detected, either alone (subtypes B, F1 and C and CRF02_AG) or in recombinant forms with subtype G and/or CRF02_AG (B, C, H and U). The introduction of different HIV genetic forms in the country is likely related with the intense population mobility motivated by emigration or with international tourism, an important economic activity in the country. The G strains circulating in Cape Verde were highly divergent when compared to the reference strains which is consistent with multiple introductions of divergent G strains from different origins [542, 561, 570, 632-634]. Indeed, we found that G variants present in Cape Verde were imported mostly from Angola and Portugal where highly divergent subtype G strains prevail [160, 625]. This was not surprising since Cape Verde has close historical, social and economical relationships with these two countries.

Viral recombination occurs in geographic areas where several subtypes and CRFs circulate simultaneously in the population. In these regions, 8-47% of the infections may be due to inter-subtype recombinants [570, 635]. In this study 30% of the viruses were recombinant and 63% of them had at least one untypable genomic fragment. Of particular interest was isolate 05CVHAN42 which is subtype C in *pol* and G in *env* a type of recombinant that has not been described before even though these two subtypes co-circulate in many African and European countries [148, 160, 625, 636]. Full-length genomic sequencing analysis will be required to determine if this isolate is indeed the first representative of a new C/G recombinant strain.

The first HIV-2 cases were identified in 1985 in individuals from Guinea-Bissau and Cape Verde [6]. However, until now, no study has characterized the genetic diversity of HIV-2 in Cape Verde. We found that all HIV-2 isolates circulating in Cape Verde were phylogeneticaly related to group A. The A group is also endemic in several West African countries related with Cape Verde such as Guinea-Bissau, Ghana, Gambia or Senegal [637-639]. Most HIV-2 viruses circulating in Cape Verde share common ancestries and are unrelated to any of the reference isolates. Interestingly, however, two isolates were found to share a close evolutionary relationship with the historic HIV-2ROD strain, the first virus to be sequenced in 1987 [640]. These results indicate that part of the long-standing HIV-2 epidemic in Cape Verde is self-renewed and suggest that the origin of the ROD isolate might have been Cape Verde [6]. In this sense, the sequences from the Cape Verde isolates are likely to provide important new insights into the origin and epidemic history of HIV-2 [73].

The recent introduction of cART in Cape Verde prompted us to investigate drug resistance mutations in isolates from treated and untreated HIV-1 infected population. We did not find *major* PI resistance mutations in either population. The *minor* mutations associated with PI resistance, L10I and A71V, were detected in two (6%) drug-naive patients. However, L10I occurs in 5-10% of untreated persons and A71V is a polymorphism that appears in 2-3% of untreated individuals. Therefore these mutations are not considered transmitted drug resistance mutations [574, 575, 631].

In contrast, we detected NRTI or NNRTI resistance mutations in three unrelated drug-naive patients infected with subtype G (05HANCV8, 05HANCV45) or a G/B recombinant (05HANCV31). M41L, present in patient 05HANCV31, confers intermediate-to-high level resistance to AZT and d4T and a lower level of resistance to ddI, ABC and TDF [631, 641]. K103N, present in patient 05HANCV08, causes high-level resistance to NVP and EFV. It has a synergistic effect with L100I on etravirine (ETR) susceptibility [631, 641]. Y318F, present in patient 05HANCV45, causes low/intermediate NVP resistance. It is a rare mutation and, unlike M41L and K103N, it is not considered a transmitted drug resistance mutation [249]. Hence, overall, we found 2 cases of transmitted drug resistance in 17 patients (12%). The finding of a moderate prevalence of transmitted HIV-1 drug resistance in Cape Verde was a surprise as the free access to cART was started officially at the end of 2004 [613]. Despite the relatively high prevalence of drug resistant strains found in treated individuals (30%), the K103N mutation was only found in one treated patient and M41L was not found in treated

patients. Hence, the most probable explanations for the origin of these resistant isolates in Cape Verde are: a) the unregulated and unmonitorized use of antiretroviral drugs previous to 2004 and b) people displacements from countries where ART is available for a longer period of time [148, 585].
Resistance mutations were detected in 3 of 10 (30%) treated patients but there was clear evidence of virological failure in only one of these patients (05HANCV55). The isolate from this patient harbored mutations conferring resistance both to NRTIs (K65R, T69d) and NNRTIs (K103N, Y181C). K65R causes intermediate resistance to ddI, ABC, 3TC, FTC and TDF, low resistance to d4T and hipersusceptibility to AZT [631, 641]. Mutations at codon 69 usually occur as T69DS/A/I/N/G and are NRTI-selected mutations [631]. T69d is a highly unusual mutation at this position. Deletions at codon 69 occur at a frequency of about 0.1% and their phenotypic and clinical significance is not known. Y181C confers high resistance to NVP and low resistance to EFV [631]. Moreover, mutations at position 181 compromise ETR response and may provide the foundation for the development of high-level ETR resistance [631]. K103N confers full resistance to EFV and NVP [438, 631]. Unfortunately we had no access to the therapeutic regimen of this patient. However, based on the resistance genotype only, this patient is a clear candidate to a PI-based second line therapeutic regimen.
In conclusion, the long-standing HIV-2 epidemic in Cape Verde is partially rooted in the ROD strain suggesting that the origin of this historical isolate was Cape Verde. The HIV-1 epidemic results from the introduction of multiple subtypes and recombinant forms by migrant workers or tourists. The prevailing and highly divergent HIV-1 subtype G has its origins in Portugal and Angola. We found a moderate prevalence of primary and secondary HIV-1 drug resistance in Cape Verde and evidence suggesting that most resistance isolates showing primary resistance mutations were imported. In the actual context of access to standardized and free therapeutic regimens, the development and implementation of drug resistance surveillance strategies in both treated and untreated population will be important to maximize the efficacy of antiretroviral therapy and prevent further transmission of drug resistance isolates in Cape Verde.

Acknowledgements

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GenBank Accession Numbers

Sequences have been assigned the following accession numbers: JF267400-JF267423 (HIV-1 *env*), JF267438-JF267463 (HIV-1 PR), JF267464-JF267490 (HIV-1 RT), and JF267424-JF267437 (HIV-2 *env*).

CHAPTER 6

Rapid clinical progression to AIDS and death in a persistently seronegative HIV-1 infected heterosexual young man

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Abstract

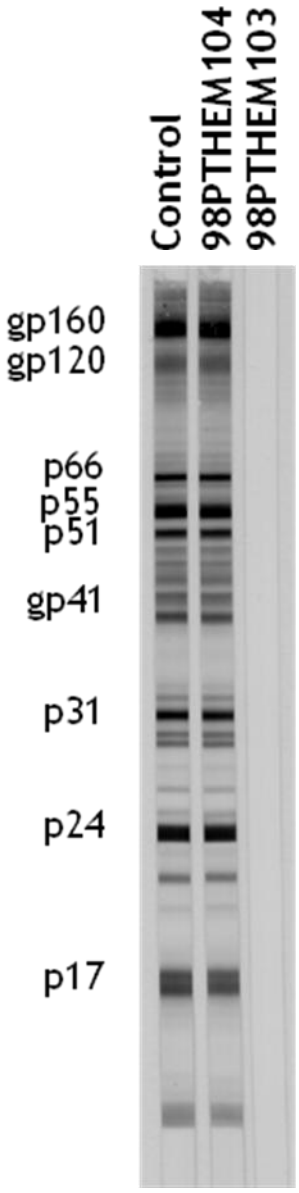
Seronegative HIV-1 infection has important implications for diagnosis and prevention. We describe a case of HIV-1 infection without seroconversion and fast progression to AIDS and death. Genetic and phylogenetic analysis indicated that the patient was infected with a CRF14_BG-like strain selectively transmitted by his seropositive sexual partner. The results suggest a massive infection with a highly aggressive CRF14_BG like strain and/or the presence of an unidentified immunological deficiency that has prevented the formation of HIV-1-specific antibodies.

HIV infection is routinely diagnosed by detection of specific antibodies in serum or plasma. This is not possible if the infected individuals do not produce HIV-specific antibodies [642-644] or in cases of infection with highly divergent HIV strains [37]. Here we present the case of a patient infected with HIV-1 CRF14_BG-like HIV-1 strain with very rapid progression to AIDS and death with persistently negative antibody tests.

Patient 98PTHEM103, a previously healthy 20-year-old heterosexual man, presented to Hospital de Santarém in September 1997 with a history of fatigue and weight loss. He had a normal lymphocyte count (3125/ml) and negative serology for hepatitis B virus, hepatitis C virus, human T cell lymphotropic virus (HTLV)-I, HTLV-II, cytomegalovirus, HIV-1 and HIV-2. On December 1997, he was diagnosed an oropharingeal candidiasis. He was still seronegative for HIV-1 and HIV-2. His total lymphocyte count was 232 cells/ml. In February 1998, after rapid clinical deterioration, he was hospitalized with fever, wasting syndrome, and oropharingeal candidiasis. Quantitative serum immunoglobulins were normal (IgA 4.7 g/l; IgM 1.5 g/l; IgG 12.2 g/l). HIV-1 and HIV-2 serology were negative by multiple enzyme immunoassay tests and western blot (Figure 6.1). His CD4 cell count dropped to 52 cells/ml. Serum p24 antigen was positive (135 pg/ml) and plasma viral load was high (>$8x10^6$ copies/ml, Amplicor HIV Monitor kit; Roche, Basel, Switzerland). He died of AIDS on March 1998. His sole HIV risk factor was a 3-month (June to September 1997) sexual relationship with patient 98PTHEM104.

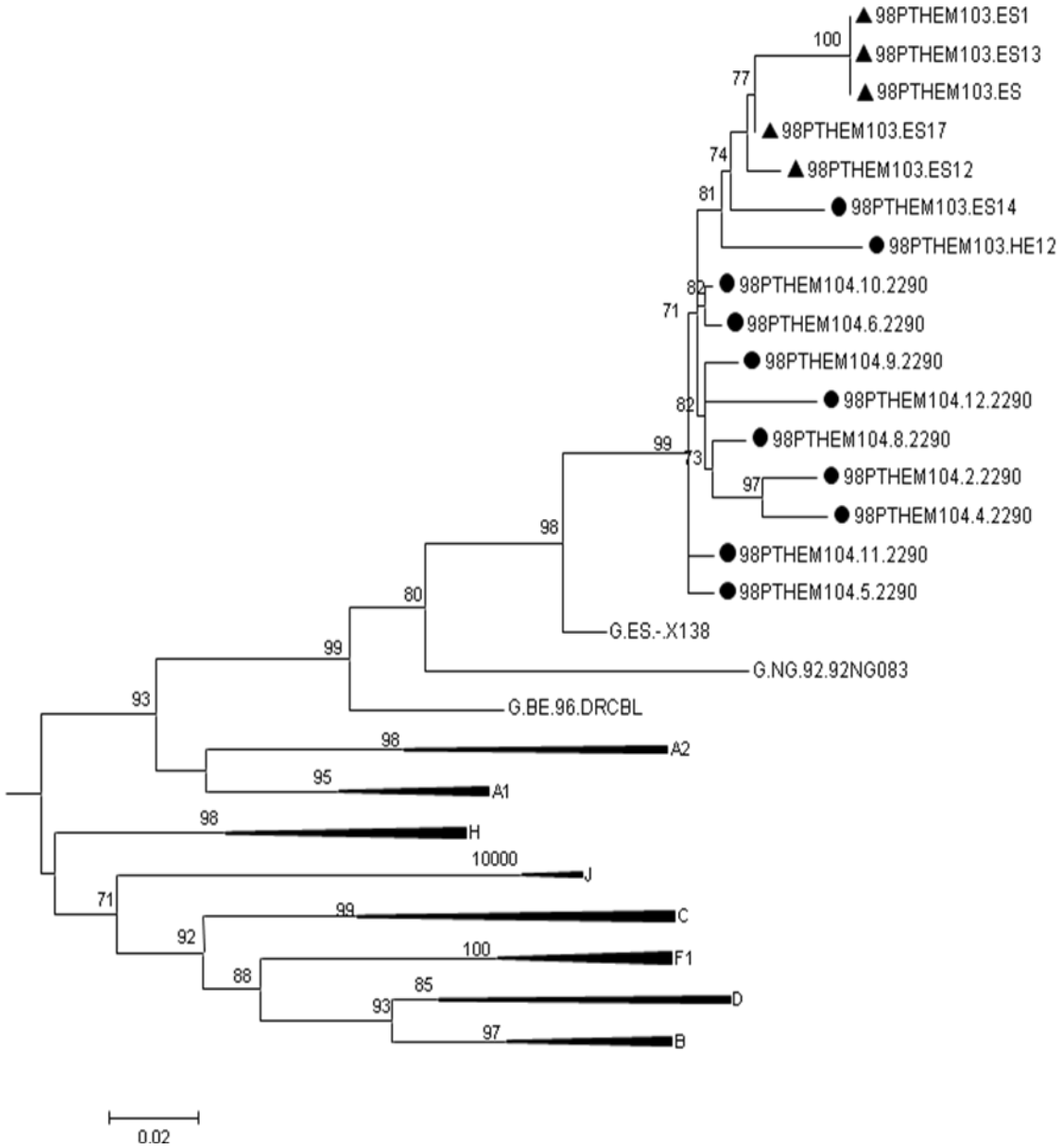
Patient 98PTHEM104, a 21-year-old woman, was an intravenous drug user and sex worker. She presented at the hospital on May 1996 with extrapulmonary tuberculosis and oropharingeal candidiasis. She tested positive for HIV (Figure 6.1). Her CD4 cell count was 40 cells/ml. In November 1996, she had an HIV-1 viral load measurement of 61 978 copies/ml and started antiretroviral therapy with indinavir, didanosine and zidovudine, which resulted in clinical and virological improvement. In September 1997, she attended the hospital with diarrhea, fever, oral candidiasis and peripheral neuropathy. She died of AIDS on February 1998.

Serological tests may yield false negative results in HIV-1 group N infections, or infection by a new and highly divergent HIV-1 variant [37]. There is also the possibility of infection by a particularly aggressive viral strain that may not permit the development of a host immune response. It is, therefore, important to analyze the genotype of the viruses from all seronegative HIV-1 infections. A nested PCR was performed to obtain a 409 bp fragment from the C2-V3 *env* region and a 582 bp fragment from the p17 *gag* region of viral isolates from both patients. Thermal cycling conditions for PCR and primers have been described previously [148]. For patient 98PTHEM103, PCR was done on chromosomal DNA extracted from sections of different postmortem tissues using an universal extraction method previously described [645]. For patient 98PTHEM104, serum collected in 1996 was the only available biologic material. Viral DNA suitable for PCR amplification was obtained by reverse transcription from RNA recovered from 200μl of serum, as described [148].



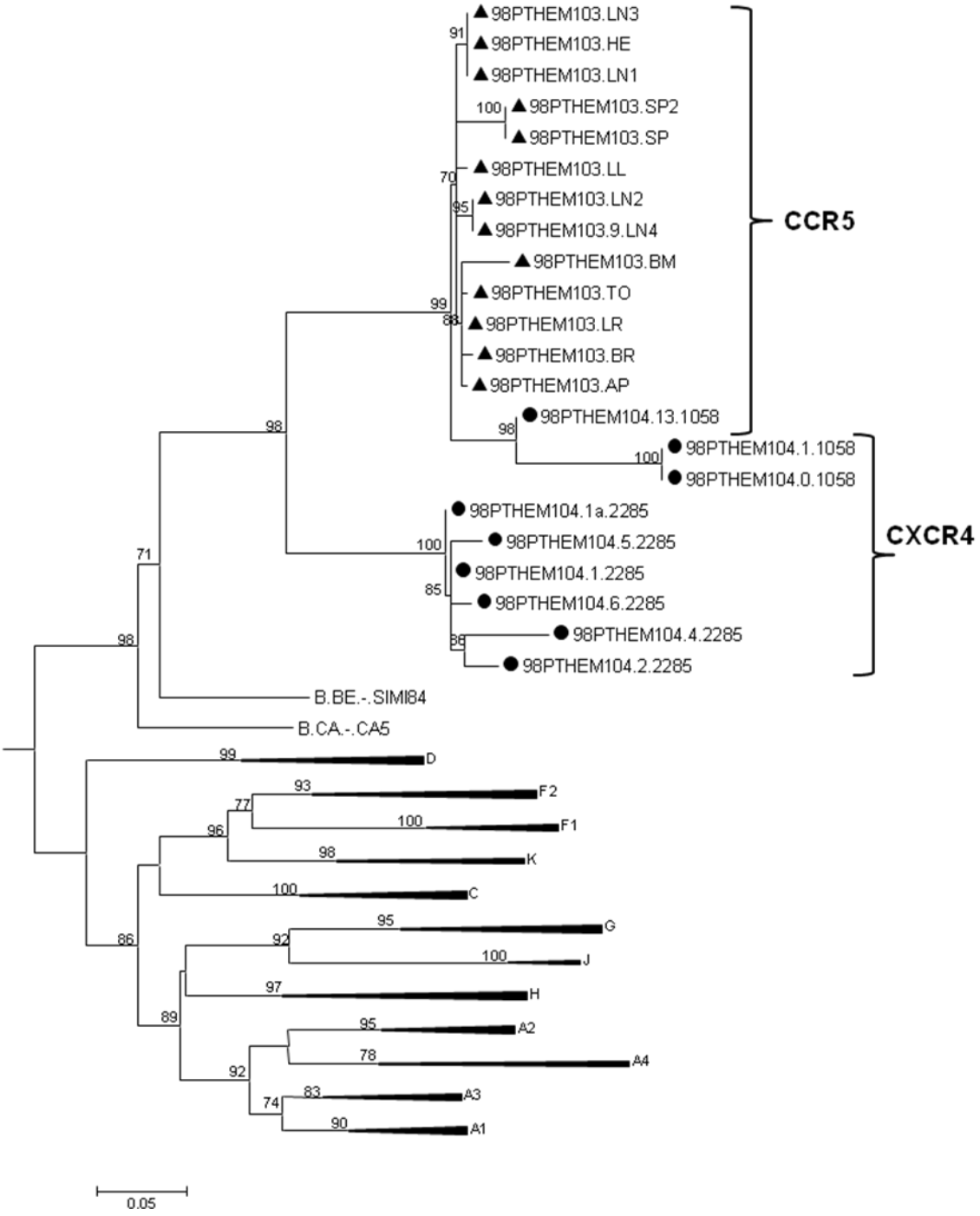
(A)

FIGURE 6.1 - Serologic and virologic analysis of patients´ 98PTHEM103 and 98PTHEM104. (A) Western blot analysis of serum samples from patient 98PTHEM103 (sample collected in December 1997) and 98PTHEM104 (sample collected in May 1996). Subtyping and evolutionary relationships between the virus isolates from these patients (bold letters) was determined by maximum likelihood phylogenetic analysis of partial C2-V3 *env* (B) and p17 *gag* sequences (C). Viral sequences from patient 98PTHEM103 were obtained from different postmortem tissues (TO, tonsil; AP, appendix; SP, spleen; BR, brain; HE, heart; BM, bone marrow; LR, right lung; LL, left lung; ES, esophage; LN, lymph nodes). The phylogenetic trees were constructed with reference sequences from all HIV-1 subtypes. In each tree, the bootstrap values supporting the internal branches defining a subtype or a sub-subtype are shown. Bootstrap values of 70% or greater provide reasonable confidence for assignment of an individual segment to one or the other genotype. The scale represents number of base substitutions per site.



(B)

FIGURE 6.1 *(continued)*



(C)

FIGURE 6.1 *(continued)*

PCR fragments were cloned into the pCR2.1 vector (Invitrogen, Carlsbad, California, USA) and sequenced. To determine virus subtype and investigate evolutionary relationships between the two isolates, maximum likelihood phylogenetic analyses was performed using the GTR+G model of nucleotide substitution, as described previously [148]. Tree searches were conducted in PAUP v4.0b10 using a nearest-neighbor interchange heuristic search strategy and bootstrap. The sequences have been assigned GenBank accession numbers GQ387120-GQ387157.

Both patients were infected with a B/G recombinant strain (B in *env* and G in *gag*) resembling HIV-1 CRF14_BG, a variant that was originally found in Portuguese and Spanish intravenous drug users (Figure 6.1) [161, 646]. Phylogenetic analysis indicated also that patient 98PTHEM104 was infected with a quasispecies evolving into two clearly separate branches, whereas patient 98PTHEM103 harbored a single virus population that was present in all tissues that were tested (Figure 6.1). Some sequences from both patients clustered together in phylogenetic analysis with high bootstrap values (Figure 6.1). This result is consistent with selective virus transmission from one patient to the other. Genotypic analysis of tropism with the Geno2pheno software [647] indicated that all virus isolates from patient 98PTHEM103 were CCR5. These results show that, in the absence of antibody pressure, CCR5 viruses may cause fatal infections and that CXCR4 viruses are not required for progression to AIDS. In contrast, only one strain from patient 98PTHEM104 was CCR5, all the others being CXCR4. Interestingly, this CCR5 strain clustered with the 98PTHEM103 strains. Sequence divergence was higher in patient 98PTHEM104 than in patient 98PTHEM103, both in the *env* (13.3% vs 1.9%) and *gag* (2.9% vs 2.4%) genes. The low genetic diversity in the virus populations from patient 98PTHEM103 is consistent with a recent infection and the absence of immunologic pressure imposed on the viruses [648]. In contrast, the higher virus genetic diversity in patient 98PTHEM104 is consistent with a longer period of infection and immune competency [649]. We therefore conclude that patient 98PTHEM104 was the index case and that she transmitted a highly virulent CCR5 virus to patient 98PTHEM103.

Rare cases of HIV-1 infected patients with clinical symptoms of AIDS but repeatedly negative for HIV antibody screening have been described [642-644]. There may be several reasons for negative HIV screening results in patients who are HIV-infected. Among host factors, the most well recognized is the window period, generally 20-25 days after infection, depending on the specific enzyme immunoassay reagents used [650]. Late seroconversions of up to 40 months after infection have been described [651, 652]. Other host factors that could explain seronegative infection include profound hypoglobulinemia, B-cell functional defects and seroreversion [332, 650]. This does not appear to be the case in patient 98PTHEM103 because he had negative results from antibody tests in all occasions and his immunological parameters were otherwise normal.

In conclusion, patient 98PTHEM103, a previously healthy individual, exhibited a fatal infection with the absence of HIV-specific humoral response. The results suggest a massive infection with a highly aggressive CRF14_BG-like strain and/or the presence of an unidentified immunological deficiency that has prevented the formation of HIV-1-specific antibodies.

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Author's contributions: N.T., R.C., and V.B. designed the research. N.T., R.C., V.B., I.B. and H.B. performed the research, interpreted the data and wrote the paper. All authors reviewed and accepted the final manuscript.

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Human experimentation and ethical guidelines of each of the authors' institutions were followed in the conduct of the clinical research described in this paper.

CHAPTER 7

Origin and epidemiologic history of HIV-1 CRF14_BG

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Abstract

Background: CRF14_BG isolates, originally found in Spain, are characterized by CXCR4 tropism and rapid disease progression. This study aimed to identify the origin of CRF14_BG and reconstruct its epidemiological history based on new isolates from Portugal.

Methodology/Principal findings: C2V3C3 *env* gene sequences were obtained from 60 samples collected in 1993-1998 from Portuguese HIV-1 patients. Full-length genomic sequences were obtained from three patients. Viral subtypes, diversity, divergence rate and positive selection were investigated by phylogenetic analysis. The molecular structure of the genomes was determined by bootscanning. A relaxed molecular clock model was used to date the origin of CRF14_BG. Geno2pheno was used to predict viral tropism.

Subtype B was the most prevalent subtype (51 sequences; 85%) followed by G (4; 7%), F1 (2, 3%), C (2; 3%) and CRF02_AG (1; 2%). Within subtype B, 6 (12%) sequences clustered with CRF14_BG reference sequences; three (50%) of those were derived from 1993 samples. Near full-length genomic sequences were strongly related with the CRF14_BG isolates from Spain. Genetic diversity of the Portuguese isolates was significantly higher than the Spanish isolates (0.044 vs 0.014, $P < 0.0001$). The mean date of origin of the CRF14_BG cluster was estimated to be 1992 (range, 1989 and 1996). Most CRF14_BG strains (15/19; 78.9%) were predicted to be CXCR4. Finally, up to four amino acids were under selective pressure in subtype B V3 loop whereas only one was found in the CRF14_BG cluster.

Conclusions: CRF14_BG emerged in Portugal in the early 1990s soon after the beginning of the HIV-1 epidemics, spread to Spain in late 1990s as a consequence of IDUs migration and then to the rest of Europe. CXCR4 tropism is a general characteristic of this CRF that may have been selected for by escape from neutralizing antibody response.

Introduction

By the end of 2009, the estimated number of adults and children living with HIV/AIDS in Portugal was 42,000 (32,000 - 53,000) [653]. The HIV/AIDS prevalence was 0.6% (0.4% - 0.7%) in the adult population, one of the highest in Western Europe [653]. After an initial period dominated by homosexual transmission of HIV-1, a shift towards transmission through heterosexual contacts and drug injection occurred and, today, heterosexual contact is the main route of HIV-1 transmission in Portugal [654]. African and Brazilian immigrants contribute substantially for the number of AIDS cases in this category [654].

The current HIV-1 epidemic in Portugal is caused by multiple subtypes, with predominance of subtype B (41.7%) followed by G (29.4%) [160, 162]. The high prevalence of these two subtypes has promoted the appearance of different types of B/G recombinant strains [161, 162, 181, 187, 188, 655]. CRF14_BG was the first epidemic CRF composed of subtypes B and G to be characterized by full-genome sequencing. This CRF was first isolated in 2002 from intravenous drug users (IDUs) in Galiza, Spain [85]. CRF14_BG displays a mosaic structure with two inter-subtype breakpoints delimiting a B subtype segment comprising most of gp120 and the 5´ half of gp41, whereas all remaining regions are classified as subtype G [85]**.** So far, only seven CRF14_BG isolates have been characterized by full-genome sequencing. These were obtained from Spanish (5/7, 71%), Portuguese (1, 14%) and German (1, 14%) IDUs patients [85, 182]. Until 2007, several sub-genomic sequences related to CRF14_BG were reported in Germany (1), Italy (2), United Kingdom (2), Estonia (15), Spain (38) and Portugal (50) suggesting that this CRF spread efficiently throughout Europe [56, 161, 162, 182, 184-195]. However, in recent years very few mentions have been made to this CRF in Europe suggesting that its prevalence has reduced significantly [655, 656]. Striking and unique features of most isolates belonging to this CRF are their CXCR4 tropism and association with rapid CD4+ T cell depletion and disease progression [193, 194, 297, 656].

To better understand the epidemiology of CRF14_BG we have characterized the full-length genome of three new CRF14_BG isolates obtained from three Portuguese patients infected in 1997, dated the origin of this CRF and reconstructed its evolutionary history. Moreover, to trace back the epidemiological history of this virus, *env* gene sequences were obtained from 60 patients infected in Portugal between 1993 and 1998. Finally, to gain some insight into the selective forces promoting CXCR4 usage by isolates belonging to this CRF, we have used genetic methods to determine the tropism of a significant number of recent Portuguese isolates and phylogenetic methods to investigate positive selection in the V3 region. Our results indicate that CRF14_BG was originated in Portugal in the beginning of the HIV-1 epidemics. From here, it probably spread to Galiza, Spain, in late 1990s and to other countries in Europe in early 2000. Our results confirm that the CXCR4 tropism is a general and stable feature of CRF14_BG and suggest that this phenotype might be a consequence of successful escape from neutralizing antibody response.

Results

Molecular Epidemiology of Partial and Near Full-Length HIV-1 Sequences

Phylogenetic analysis showed that HIV-1 C2-C3 sequences belonged to different subtypes (Figure 7.1). As expected, B was the most prevalent subtype (51 sequences; 85%) followed by subtypes G (4; 7%), F1 (2; 3%), C (2; 3%) and CRF02_AG (1; 2%). Within subtype B, 6 (12%) sequences clustered with the CRF14_BG reference sequences. Importantly, 3 (50%) of these sequences (EU335936, EU335937 and EU335940) were obtained from samples collected in 1993. These results suggest that CRF14_BG was already circulating in Portugal in 1993.

Near full-length genomic sequences were obtained for three isolates from three HIV-1 infected patients residing in Lisbon. These were two children (00PTHSM5, 00PTHDE10) infected by vertical transmission in 1997 and one young adult (98PTHEM103) infected by heterosexual contact in the same year (Table 7.1) [297]. Phylogenetic analyses revealed that the sequences were strongly related with reference CRF14_BG isolates from Spain (Figure 7.2). Bootscan analyses revealed that the new isolates share a mosaic structure that is similar to the reference CRF14_BG strains with only two intersubtype breakpoints delimiting a B subtype segment comprising most of gp120 and the 5´ half of gp41 and the remaining portions of the genome of subtype G (Figure 7.3).

CRF14_BG Was Originated in Portugal in Early 1990s

The mean date of origin of the CRF14_BG cluster was estimated to be 1992 (range 1989 and 1996). The Portuguese CRF14_BG full genome sequences were not monophyletic, but clustered with Spanish CRF14_BG sequences. Therefore, no discrimination could be made between the time of entry of this CRF in Portugal and in Spain. Notably, two full-length subtype G sequences from Spain (G.ES.00.X558 and G.ES.99.X138) clustered within the CRF14_BG cluster, indicating a possible subtype G ancestor for this CRF. On the other hand, the divergence rate of the Portuguese isolates was higher than the Spanish isolates (0.030 substitutions/site/year vs 0.024). Likewise, the genetic diversity between Portuguese isolates was significantly higher than between the Spanish isolates (0.044 vs 0.014, $P < 0.0001$). Taken together, these results are indicative of a longer evolutionary time of the Portuguese strains and suggest, therefore, that CRF14_BG was originated in Portugal rather than in Spain.

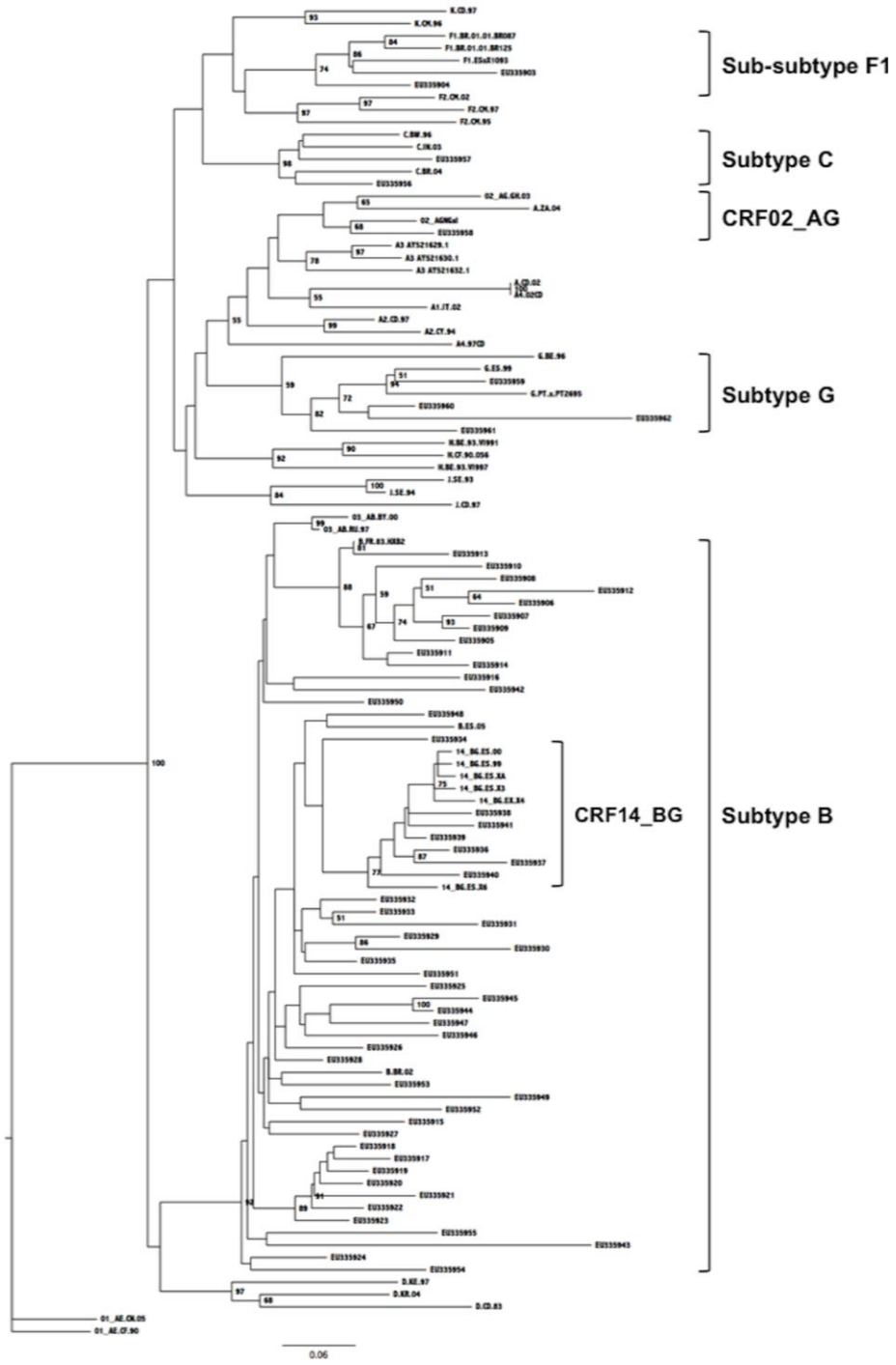

FIGURE 7.1 - Phylogenetic analysis of *env* gene sequences from HIV-1 infected patients. The maximum likelihood phylogenetic trees were constructed with reference sequences from all HIV-1 subtypes. The bootstrap values supporting the internal branches defining a subtype or a CRF are shown. Bootstrap values of 70% or greater provide reasonable confidence for assignment of an individual segment to one or the other genotype. The scale represents number of base substitutions per site.

TABLE 7.1

Epidemiological Characterization of CRF14_BG Infected Patients

Sample	Gender	Ethnic group	Year of birth	Transmission route	Year of diagnosis	Genebank accession number
PTHSM5	F	Caucasian	1997	MTCT	1997	GU230138
00PTHDE10	M	Caucasian	1997	MTCT	1997	GU230137
98PTHEM103	M	Caucasian	1978	Heterosexual[a]	1998	GU230139

MTCT - mother to child transmission; [a] Individual infected by sexual contact with HIV infected female sex worker which was intravenous drug user [297].

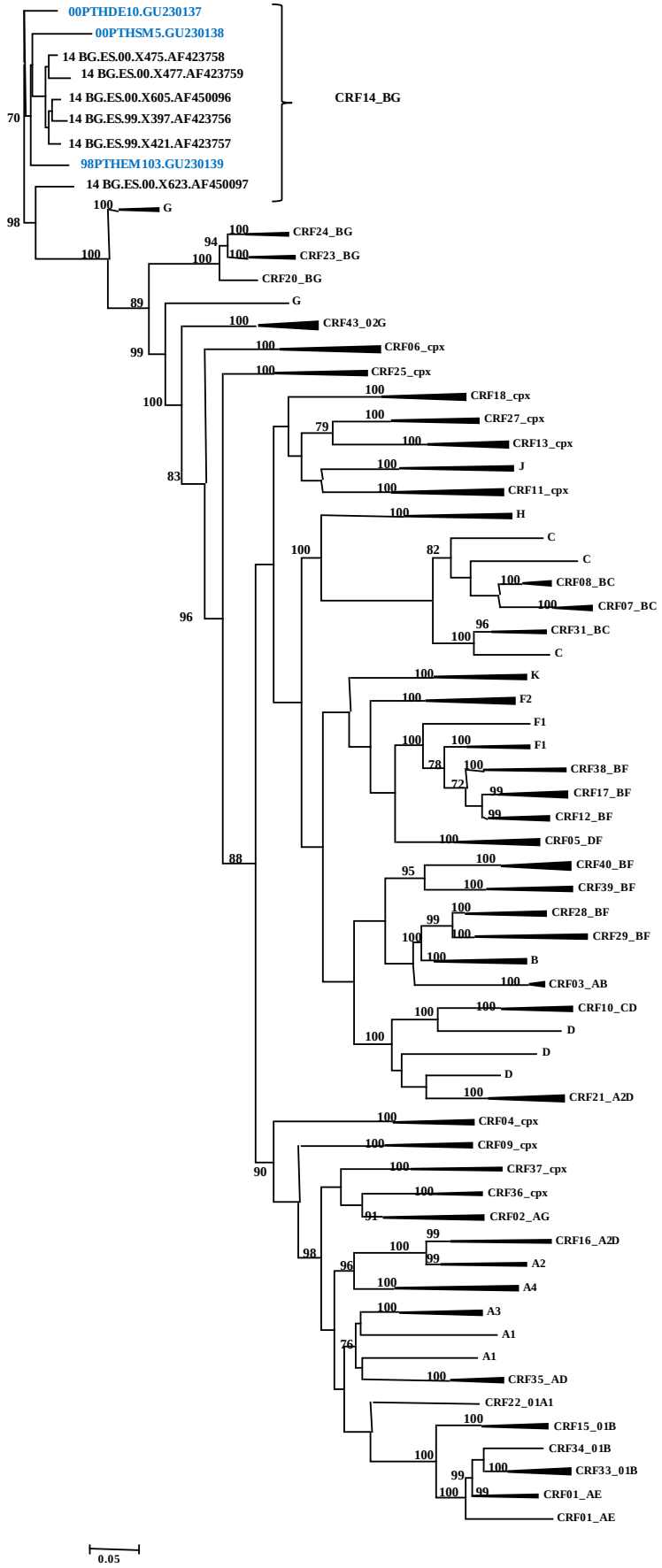

FIGURE 7.2 - Phylogenetic analysis of full-length genomic sequences from HIV-1 infected patients (blue). The maximum likelihood phylogenetic trees were constructed with reference sequences from all HIV-1 subtypes. The bootstrap values supporting the internal branches defining a subtype or a CRF are shown. Bootstrap values of 70% or greater provide reasonable confidence for assignment of an individual segment to one or the other genotype. The scale represents number of base substitutions per site.

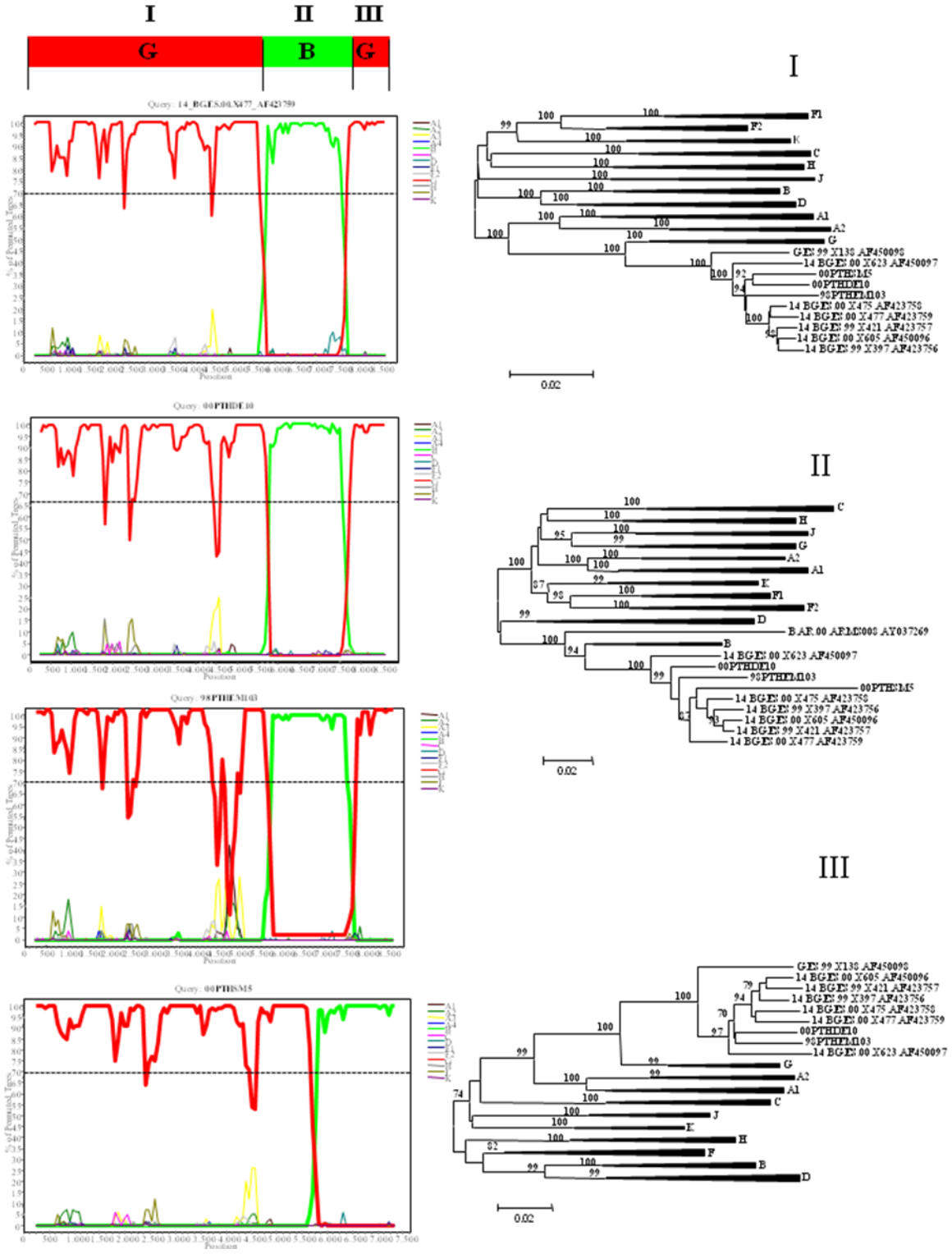

FIGURE 7.3 - Bootscanning analysis of full-length genomes from Portuguese and Spanish CRF14_BG isolates. The dashed line indicates the cut-off of 70%.

Most CRF14_BG Isolates Use CXCR4

Geno2pheno predicted that most (15/19; 78.9%) recombinant G *pol*/B *env* sequences (corresponding to the full-genome CRF14_BG sequences recombination pattern) used CXCR4, while only four used CCR5. Notably, the phylogenetic tree of the recombinant BG sequences and control subtype B sequences from the Portuguese and Los Alamos Database indicated a cluster of CXCR4 using sequences that included 14 of the BG sequences that used CXCR4 together with 3 other subtype B control sequences that also used CXCR4 and only 2 CCR5 using BG recombinants (cluster had 19 sequences, of which 17 used CXCR4, 89.5%, LRT value of the cluster=0.98). If we extend the cluster backwards, we find a 25 sequences cluster (subtype B and recombinant BG) of which 23 use CXCR4 (92%, LRT=0.82). In no other cluster of the tree did we identify such a big proportion of CXCR4 using strains, indicating that there may be something innate in these sequences that make them evolve to using CXCR4 more frequently than other sequences (Figure 7.4).

Positive Selection Might Explain the Evolution to CXCR4 Usage in CRF14_BG Isolates

We analyzed selective pressure both in the full tree and in the BG recombinants cluster. The selective pressure analysis of the complete tree consistently identified in the two models positive selection in amino acid 11 of the V3 loop, which is a main determinant of co-receptor usage (Table 2) [657, 658]. Amino acids 20 and 35 were also consistently identified as being positively selected. Furthermore, the SLAC method also indicated amino acids 21 and 23 of V3 as being under positive selective pressure and the dual variable rates model further indicated amino acid 26. When we analyzed only the BGs subtree (19 sequences), we found no evidence of positive selection in V3 when using the SLAC model while when using the dual variable rates model the amino acid 22 was indicated as being positively selected (Table 7. 2).

Discussion

We provide new molecular and epidemiologic evidence suggesting that CRF14_BG emerged in Portugal in the early 1990s soon after the beginning of the HIV-1 epidemic. This was surely a direct consequence of the early co-circulation of subtypes B and G among the HIV-1 infected population. In fact, we show here that three CRF14_BG-like isolates were already present in Lisbon in 1993. Definitive proof of the early presence of CRF14_BG in Portugal was obtained by genomic sequencing of three isolates obtained in 1997 from patients representing the two most important transmission groups (vertical and heterosexual transmission). Molecular clock analysis indicated that the ancestor of the Portuguese CRF14_BG viruses dates back to 1992. The early presence of CRF14_BG in these transmission groups implies that it was rapidly converted into a highly successful epidemic strain.

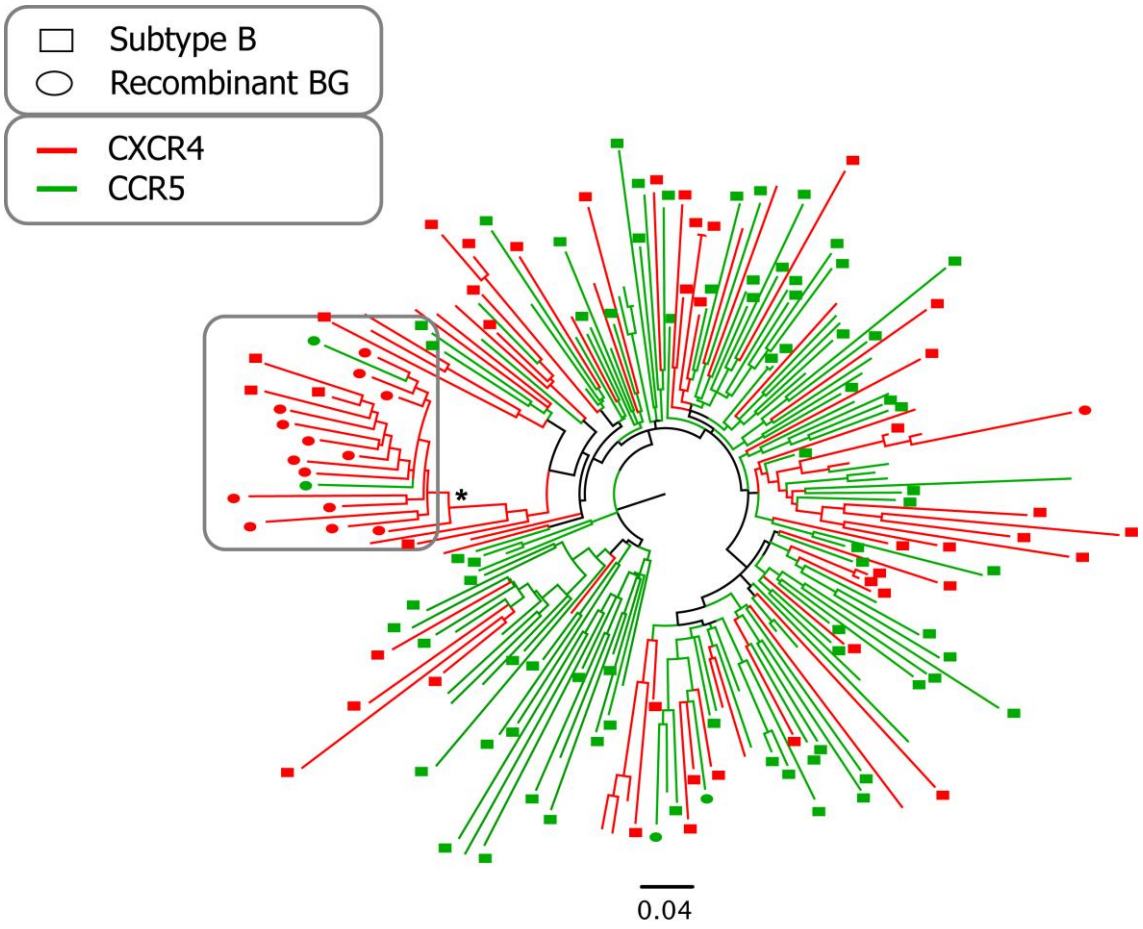

FIGURE 7.4 - Phylogenetic tree for the C3-V3 genomic region of the recombinant BG sequences, sequences downloaded from the Los Alamos Database and from Portuguese patients, showing the significant clustering of BG sequences and associated CXCR4 usage. Sequences are labeled according to subtype and coreceptor usage. Sequences were either subtype G in *pol* and subtype B in *env* (circles), subtype B both in *pol* and in *env* (squares). If the sequences were subtype B only in *env* (no information available for *pol*), no label was added. Sequences colored green use CCR5 only, while sequences colored red can use CXCR4. Asterisk indicates significant support for the cluster (LRT > 0.95). Tree was built as described in the methods section.

TABLE 7.2

Positive Selective Pressure on the V3 Loop of the Analyzed Dataset

Method	Codons under selective pressure[1]	
	Whole Tree (Bs+BGs) (Number of sequences in cluster = 201)	BGs cluster (Number of sequences in cluster = 19)
SLAC	11, 20, 21, 23 and 35	None
Dual variable rates	11 , 20, 26 and 35	22

[1]Codons identified by Hyphy as being significantly (P<0.05) under selective pressure are indicated.

CRF14_BG was found in Galiza, Spain, in 2002 among HIV-1 infected IDU patients of Spanish (5 patients) and Portuguese (1) origin [85]. Between 1999 and 2007 CRF14_BG-like strains were found abundantly in Portugal, Spain and other European countries [56, 160-162, 182, 184-187]. In Portugal, in 2003, CRF14_BG prevailed over all other recombinants [161, 187]. Since then, however, CRF14_BG prevalence decreased significantly in Portugal [160, 655] and Spain [656] and, to our knowledge, it has not been reported elsewhere in the world. One reason for this decrease in prevalence of CRF14_BG might be related with its high tendency for recombination with other subtypes or recombinant forms. This is suggested by the multiple CRF14_BG-like sub-genomic fragments that have been described in the recent literature [191, 655] and by the existence of at least three other BG intersubtype CRFs (CRF20_BG, CRF23_BG and CRF24_BG) [196]. Alternatively, CRF14_BG prevalence may have decreased due to its unusually high pathogenicity. We show here that most CRF14_BG isolates circulating in Portugal form a single cluster and use the CXCR4 co-receptor. The majority of CRF14_BG isolates from Spain also use CXCR4, even those obtained from patients at early stages of infection [193, 194, 656]. In subtype B infected subjects, baseline infection with a CXCR4-using virus is strongly associated with a greater decrease in CD4+ T cell count over time and a greater risk of disease progression [659-661]. Consistent with this, a rapid decrease in CD4+ T cell counts has been observed in all patients infected with CRF14_BG isolates [194]. Moreover, we have shown recently that CRF14_BG infected patients can progress very quickly to AIDS and death [297]. Taken together, these results provide strong argument to suggest that, like HIV-1 subtype D, CRF14_BG may be highly pathogenic [298, 299].

We show that positive selection acts differently in the V3 loop of CRF14_BG isolates compared to B isolates. In fact, between 0-1 amino acids are under selective pressure in CRF14_BG V3 loop whereas in subtype B these are 3-4. Of particular interest in this context was the finding that amino

acid 11 in the V3 loop, which is a main determinant of co-receptor usage [657, 658], was not under selective pressure in the CRF14_BG cluster of viruses. These findings suggests that strong conformational and/or functional constrains prevent changes in the V3 loop of this CRF and implies that the CXCR4 tropism is a stable phenotypic feature of CRF14_BG isolates. Neutralizing antibodies are the main selective forces acting on the HIV-1 envelope and escape from these antibodies can promote rapid envelope evolution [662, 663]. CXCR4 tropism has been associated with escape from neutralizing antibody response both in HIV-1 infection and HIV-2 [664-666]. Hence, CXCR4 tropism in CRF14_BG might have been a direct consequence of successful escape from neutralization in infected subjects. In this context, it is important to note that the only R5 CRF14_BG isolate described so far was found in a individual that progressed to AIDS and death in only seven months without producing HIV antibodies (seronegative infection) [297].

In conclusion, CRF14_BG probably emerged in Portugal in the early 1990s soon after the beginning of the HIV-1 epidemics and spread to Galiza, North of Spain, in late 1990s as a consequence of the mobility of HIV-1 infected IDUs. Until 2007 CRF14_BG spread efficiently in Europe and elsewhere and from then on there was a significant decrease in its detection. CXCR4 tropism is a unique characteristic of this CRF that may have been selected for by escape from neutralizing antibody response. The reasons for the current low prevalence of this CRF remain unknown but may be related with high recombination rate with other subtypes or recombinant strains and/or with unusually high virulence and pathogenicity.

Methods

Sample Collection and Sequencing

HIV-1 blood samples were collected from 60 HIV-1 patients infected between 1993 and 1998 in the North (Porto) and South (Lisbon) of Portugal. Viral genomic RNA was extracted from plasma and reverse transcribed. A nested PCR technique was used to amplify a 409 pb HIV-1 C2-C3 *env* region as described elsewhere [548]. PCR products were sequenced using the BigDye Terminator Cycle sequencing kit (Applied Biosystems), and an automated capillary sequencer (ABI PRISM 310, Applied Biosystems). Three patients residing in Lisbon, two children infected by vertical transmission and one adult infected by heterosexual contact, all infected in 1997, were selected for full-length genomic sequencing (Table 7.1). For this study, chromosomal DNA was extracted from *post-mortem* tissue (patient 98PTHEM103) or from peripheral blood mononuclear cells (00PTHSM5, 00PTHDE10) using a universal extraction method as described elsewhere [645]. Full-genome PCR amplification and sequencing was done as described elsewhere [74].

Subtyping of HIV-1 Sequences

The genomic sequences were aligned with reference sequences obtained from the Los Alamos HIV Sequence Database using *Clustal-X* [195] and manual adjustments were made using *Genedoc* [667].

To confirm recombination events and identify recombination breakpoints, bootscanning analysis was performed using *Simplot 3.5.1* [556]. Maximum likelihood analyses [551] were performed using the best-fit models of molecular evolution as estimated by *Modeltest* under the Akaike information criterion [552]. These were the TVM model for the full-genome sequences and TVM+G+I for the C2-C3 sequences. Tree searches were conducted in *PAUP v4.0b10* using a nearest-neighbor interchange heuristic search strategy and bootstrap.

Dating the Origin of CRF14_BG

To date the origin of CRF14_BG, a non-recombinant region of the genome was used (1442 – 4281 bp relative to HXB2). This is a subtype G genomic region. Fourteen taxa collected from the Los Alamos Database (http://www.hiv.lanl.gov/) were aligned to our three new full-genome CRF14_BG sequences. The Los Alamos sequences included four CRF14_BG sequences from Spain and Denmark (14_BG.DE.01.9196_01, 14_BG.ES.00.X605, 14_BG.ES.00.X605 and 14_BG.ES.99.X397) as well as 10 subtype G sequences (G.ES.00.X558, G.ES.99.X138, G.BE.96.DRCBL, G.CM.01.01CM_4049HAN, G.CM.96.96CMABB55, G.FI.93.HH8793_1_1, G.NG.92.92NG083, G.NG.x.01NGPL0669, G.NG.x.01NGPL0760 and G.SE.93.SE6165). The model of evolution used was HKY85 with a 4 class gamma distribution to model rate variation among sites and allowing for a proportion of invariable sites. A relaxed molecular clock model implemented under a flexible demographic model (Bayesian skyline plot) was used to date the origin of CRF14_BG as described previously [430]. A prior uniform distribution was set for the date of origin of the phylogeny with a uniform interval between 1901 and 1998. Two BGs clusters – one including only Portuguese CRF14_BG sequences and another including both Portuguese and Spanish CRF14_BG sequences – were defined. These were given prior distributions for the root of the clade between 1931 (the date of origin of HIV-1 as published by Korber et al [43]) and 1998 (1998 was the date of sampling of the oldest sampled CRF14_BG sequence, indicating that the recombinant certainly existed in that year).

Co-receptor Usage, Selective Pressure and Divergence Rates Estimation

Pairwise genetic distances and divergence rates of Portuguese and Spanish CRF14_BG isolates were calculated as described previously [668].

For coreceptor usage analysis, we included new partial *env* sequences in our alignments. The alignments now spanned the C3-V3 region and included 201 sequences. These were sequences collected either from the Los Alamos Database or sequences from Portuguese patients collected for the purpose of coreceptor usage determination before starting Maraviroc treatment [655]. If a patient had a subtype G sequence from *pol* and a subtype B sequence from *env,* it was classified as a BG recombinant (circles in Figure 7.4). If a patient had a subtype B sequence both in *pol* and *env*, it was classified as a pure subtype B (squares in Figure 7.4). For the patients collected from the Los Alamos Database, sometimes there were only subtype B sequences in *env;* these sequences were left unclassified (taxa not marked with circles nor squares in Figure 7.4). In total, 201 sequences

were included in the alignment, of which 19 corresponded to BG recombinants, 114 were pure subtype B (subtype B in *pol* and *env*) and the remaining were Los Alamos subtype B sequences only in *env* (subtype B in *env*, but unknown subtype for *pol*).

Co-receptor usage prediction of each sequence was made using the *geno2pheno* software [647], after codon-aligning the C3-V3 genomic region with the *GeneCutter* tool available at the Los Alamos website (http://www.hiv.lanl.gov/content/sequence/GENE_CUTTER/cutter.html).

The estimation of the underlying phylogenies for the estimation of selective pressure was made with the *PhyML* software, using the HKY85 substitution model with gamma distributed rate variation and a proportion of invariant rates. The tree improvement was made using the subtree-pruning regrafting (SPR) heuristic search. The reliability of each cluster was determined using the likelihood ratio test (LRT) method as implemented in *PhyML*. Finally, site-by-site selective pressure was calculated using different models available at the *HyPhy* package. We started by using the simple single likelihood ancestor counting method (SLAC), a counting method that employs maximum likelihood ancestral reconstructions. Then, we applied a more complex dual variable rates model, with the MG94xHKY85 codon rate matrix and dual variable dS and dN rates drawn from bivariate independent discrete distributions, with 4 rate classes.

Statistical Analysis

Statistical analysis was performed in *GraphPad Prism version 4.0* for Windows (GraphPad Software), with a level of significance of 5%. Pairwise genetic distances and divergence rates were compared using the Mann Whitney U test.

GenBank Accession Numbers

Sequences have been assigned GenBank accession numbers GU230137 - GU230139 (full-length genomic sequences) and EU335962 - EU335903 (C2-C3 sequences).

Author Contributions

NT and RC designed research. IB, AB, PB, HB, FM and PG performed research. IB, AB, FM, RC and NT interpreted the data. IB, AB and NT wrote the paper. All authors reviewed and accepted the final manuscript.

CHAPTER 8

General Discussion and Conclusions

This work documents the genetic diversity of HIV in patients from Angola, Mozambique, Cape Verde and Portugal, countries that have strong historical, social, cultural and economical ties and very different HIV-1 epidemics. The origin and epidemiological histories of HIV-1 subtypes and CRFs circulating in these countries were investigated as well as their potential impact in diagnosis, disease progression and susceptibility to antiretroviral therapy. Finally, the nature, dynamics and prevalence of primary drug resistance mutations was determined in untreated patients from Angola, Mozambique and Cape Verde.

In Angola we found an extremely high genetic diversity among HIV-1 strains (Chapter 2). Indeed, we could not amplify HIV-1 DNA from 52 samples possibly due to the extremely high genetic diversity of HIV-1 [52, 159, 312]. In the 159 samples that we were able to amplify and sequence, we found all HIV-1 group M subtypes and sub-subtypes as well as several CRFs and URFs. Subtype A and its sub-subtypes were the predominant HIV-1 genetic forms (13.8%), followed by subtype C (11.3%). This was not surprising because the countries in the Northern border of Angola have a predominance of subtype A [159, 543, 560, 561] while in the Eastern border subtype C prevails over the other subtypes [562]. Of note was the relatively frequent detection of the rare H and J subtypes and almost 8% of U sequences.

Some isolates formed distinct sub-clusters within the subtype A radiation (Figure 2.1) and their genetic distances were similar to the ones observed within the other A sub-subtypes for the same genomic regions [48]. Hence we propose that these sequences may define new A sub-subtypes.

In regions where several HIV-1 genetic forms circulate recombination can be very frequent (up to 24% of the infections) [48, 565]. Consistent with this, and with the remarkable HIV-1 diversity in Angola, we found that almost 50% of the viruses were recombinants (Chapter 2). The first generation recombinant strains were in general extremely complex (composed of multiple subtypes and U sequences) and there were also 36% of second-generation recombinants.

Remarkably, we found that a number of our sequences fall at basal positions on the phylogenetic trees and that some strains have little organized substructure and form weaker clusters within phylogenetic trees than the global reference sequences, not allowing a clear distinction between subtypes. As a consequence, the current global subtype classification may not reflect the extent of diversity in Angola [35]. Further analyses with genomic sequences will be needed to clarify whether the Angolan strains are ancestor viruses, pure subtypes or represent new genetic forms of HIV-1.

The available epidemiologic data [537] and the very high intra-subtype divergence of Angolan HIV-1 isolates are consistent with a long-standing HIV-1 epidemic in this country [50, 51, 91, 92, 100, 159, 541, 543, 561, 566]. We speculate that the colonial war with the Portuguese (1961-1974) was the major event contributing to the early dissemination of HIV-1 in the country. Indeed, during the first year of the colonial war, many Angolans migrated to the neighboring countries (mainly DRC and Republic of Congo). Upon return to their home country, at the end of 1961 beginning of 1962, they

probably brought with them the different genetic forms of HIV-1 that have been present in those countries since the 1940s [17, 41, 42, 50, 51, 92, 159, 543, 560, 561, 566].

Others and we have found that the HIV-1 epidemic in Portugal [122, 160-162, 181, 186-188, 669] (this thesis, Chapter 7) and Cape Verde (this thesis, Chapter 5) is caused by a high proportion of non-B subtypes and recombinant forms, some of which are closely related with the Angolan isolates. Hence, the intense people displacements during the colonial war with Angola may also have promoted the dissemination of HIV-1 non-B subtypes from Angola to Portugal and Cape Verde, possibly in the mid-1960s, and from there to the rest of the world. In this regard, Angola may have been one of the epicenters of the current HIV-1 group M worldwide epidemics [17, 42].

In Maputo, Mozambique, most patients (80.8%) were infected with HIV-1 subtype C which, together with previous studies, demonstrates that this is the prevailing subtype in Maputo [147, 599, 600] (Chapter 4). The subtype C sequences were spread across multiple clusters containing other subtype C sequences from neighboring countries, which provides evidence in favour of multiple introductions of this subtype in the country, and against a specific Mozambican subtype C cluster. These results are consistent with previous studies demonstrating the existence of many subtype C sub-lineages in Southern Africa [607]. Remarkably, in contrast to other studies, in Maputo we also detected all other major non-B subtypes and some untypable variants. Of particular relevance in this context, was the finding of a cluster of seven closely related CRF37_cpx isolates that we think were recently imported into the country [180]. In all, our results suggest that the HIV-1 epidemic in Maputo is evolving from a monotypic subtype C epidemic to one that is caused by multiple and complex genetic forms. It will be important in the future to determine in what way this changing viral diversity in Maputo impacts the HIV/AIDS epidemic in Mozambique.

In Cape Verde we characterized for the first time the circulating genetic forms of HIV-1 and HIV-2 (Chapter 5). Almost 50% of the patients were infected with HIV-1 subtype G; along with other subtypes we also found some U variants, alone or in recombinant forms. The G strains circulating in Cape Verde were highly divergent when compared to the reference strains which is consistent with multiple introductions of this subtype from different origins [542, 561, 570, 632-634]. One of the most likely origins of great part of the G variants present in Cape Verde should be Angola and/or Portugal [160, 625]; this is not surprising since Cape Verde has close historical, social and economical relationships with these two countries. It will be interesting to date the introduction of subtype G in the country and see whether the current epidemic is in any way related with the displacement of soldiers to Angola during the colonial war with the Portuguese (see above).

Of particular interest was the finding of a C(*pol)*/G(*env)* recombinant, because this type of recombinant had not been described before even though these two subtypes co-circulate in many African and European countries [148, 160, 625, 636]. Full-length genomic sequencing analysis will be required to determine if this isolate is indeed the first representative of a new C/G CRF.

All HIV-2 isolates circulating in Cape Verde were from group A (Chapter 5). Group A is also endemic in Portugal and several West African countries related with Cape Verde [637-639]. Remarkably, we

found two viruses sharing a close evolutionary relationship with the historic HIV-2 ROD strain, the first HIV-2 to be sequenced back in 1987 [640]. This result strongly suggests that Cape Verde was the country of origin of HIV-2 ROD and put this country at the epicenter of the worldwide HIV-2 epidemics. In this regard, the new HIV-2 sequences from Cape Verde may provide important new insights into the epidemic history of this virus [73].

In Chapters 3-5, we have also characterized the type and number of drug resistance mutations in isolates from untreated HIV-1 infected patients some treated patients from Cape Verde were also included), as well as their predicted susceptibility to the drug regimens available in these countries. Additionally, we tried to determine the origin of the resistant isolates.

We did not find major PI drug resistance mutations in HIV-1 strains from Angola, Mozambique and Cape Verde. This is consistent with the fact that the first line ART regimens available in these countries does not include PI [148, 256, 572, 580]. However, there were numerous natural polymorphisms in the PR of most non-B subtypes from these countries that have been considered as minor PI resistance mutations in subtype B. Selected examples are L10I/V (found in 17.7% of the isolates in Angola), V11I (detected in 4.3 % of subtype A infected patients in Angola) and T74P. L10I/V has been associated with resistance to most of the PIs when present with other mutations [485, 576]. V11I and T74P has been associated with resistance to darunavir [485, 486], furthermore T74P was also considered a major tipranavir resistance mutation in the RESIST study [576]. The frequencies of L10I/V, V11I and many other known polymorphisms associated with drug resistance was significantly higher in our patients than in other untreated patients infected with the same subtypes whose *pol* sequence has been deposited in the Stanford HIV Drug Resistance Database [670]. Furthermore, we found some new polymorphisms in Angolan, Mozambican and Cape Verdean viruses that also have not been previously described in the referred Database. Overall, these results confirm that the viruses circulating in these countries are highly divergent and suggest that they may have a low genetic barrier to resistance to some PIs.

In the RT we also found evidence of numerous new polymorphic sites that may impact the susceptibility to RT inhibitors (Chapters 3-5). Most importantly, we found some drug resistance mutations in untreated individuals from the three countries. However, the prevalence of TDR varied significantly between countries. Whereas in Angola there was a low prevalence of TDR (1.6%) that is similar to the levels for other Sub-Saharan African countries where ART therapy is still not widely available [254, 258, 259, 511, 577, 583, 584], in Maputo (5.9%) and Cape Verde (12%) there was a moderate level of TDR. The mutations associated with TDR were similar in all countries: M41L, D67N, M184V, L210W, T215Y/F, K219Q and K103N. These mutations are typically selected by the first line ART regimens available in these countries (based on NNRTIs and NRTIs) and are among the most frequently TDR mutations detected worldwide [249]. Importantly, analysis of predicted drug susceptibility indicated that none of the isolates containing these mutations would be fully sensitive to the standard first line ARV regimens used in Angola, Mozambique, and Cape Verde.

The low prevalence of TDR found in Angola at the time of the study (1993-2001) is consistent with the very restricted access to ART in those days, aggravated by the Angolan civil war that lasted until 2002. However, the finding of a moderate level of TDR, in Mozambique and especially in Cape Verde was unexpected due to the restricted availability of antiretroviral drugs until 2001-2004. The two most likely explanations for the origin of the resistant isolates in Mozambique and Cape Verde are the unregulated and unmonitored use of ART drugs bought in the black market or abroad [585], and displacements of people from countries where ART has been available for a longer period of time [583]. In Mozambique and Cape Verde we found some epidemiologic and phylogenetic evidence suggesting that the resistant viruses were indeed imported. Nonetheless, we can't formally exclude the possibility that the supposedly untreated patients harbouring DRMs used ARV drugs on their own. Future research on TDR in these countries should be performed on recently infected patients, as this is the only way to guarantee that the patients are indeed naive to therapeutics. In the actual concerted action effort to provide generalized access to standardized and free ARV therapy, the development and implementation of feasible drug resistance surveillance strategies in both treated and untreated populations should be regarded as a high priority in these countries. A rationally designed national drug resistance surveillance system is currently regarded as pivotal to maximize the long term efficacy of standardized ART regimens, prevent accumulation of DRMs and prevent transmission of drug resistance isolates [671].

In Portugal we studied a rare case of seronegative HIV-1 infection with extremely rapid progression to AIDS and death (Chapter 6). It is important to analyze the virus genotype from all seronegative HIV-1 infections, since serological tests may yield false negative results with new and highly divergent HIV-1 variants [37]. There is also the possibility of infection by a particularly aggressive viral strain that may not permit the development of a host immune response. We found that the seronegative HIV-1 patient was infected with a B/G recombinant strain (B in *env* and G in *gag*) resembling HIV-1 CRF14_BG, a variant that was originally found in Portuguese and Spanish IDUs [161, 646]. Phylogenetic analysis showed that he got his virus from a female contact who was an IDU, and there was a very restrict genetic evolution of the virus (genetic distance between variants found in different *post mortem* tissues was very low). Genotypic analysis of tropism indicated that he was selectively infected with a CCR5 variant. These results show that in the absence of antibody pressure CCR5 viruses evolve very slowly and may cause rapid progression to AIDS and death. Overall, our results suggested a massive infection with a highly aggressive CRF14_BG-like strain and/or the presence of an unidentified immunological deficiency that prevented the formation of HIV-1-specific antibodies.

Finally, by sequencing and phylogenetic analysis of the viral genome present in three unrelated B/G infected patients, we provide the first molecular and evolutionary characterization of the CRF14_BG strains circulating in Portugal (Chapter 7). We found new molecular and epidemiologic evidence suggesting that CRF14_BG emerged in Portugal possibly in 1992 (range, 1989 and 1996) soon after the beginning of the HIV-1 epidemic, and from there it spread to Galiza, Spain, and then to the rest

of the world via, most likely, HIV-1 infected IDUs. Currently, it seems however, that CRF14_BG prevalence has decreased significantly [160, 655, 656]. One reason for this decrease might be related with its high tendency for recombination with other subtypes or recombinant forms [191, 655]. This is suggested by the existence of at least three other BG intersubtype CRFs (CRF20_BG, CRF23_BG and CRF24_BG) [196]. Another explanation for the apparent decrease in the prevalence of CRF14_BG could be related with its unusually high pathogenicity. In fact, by causing severe immune deficiency and death in a short period of time, its window of transmissibility between hosts would be very short and its prevalence could decrease significantly in a few years. Indeed, a review in the literature shows that most CRF14_BG isolates described to date, like most HIV-1 subtype D isolates [298, 299], use the CXCR4 co-receptor and are associated with rapid CD4 depletion and disease progression [193, 194, 297, 656]. In addition, we found that positive selection acts differently in the V3 loop of CRF14_BG isolates compared to B isolates. Of particular interest in this context was the finding that amino acid 11 in the V3 loop, which is a main determinant of co-receptor usage [657, 658], was not under selective pressure in the CRF14_BG cluster of viruses. These results suggests that strong conformational and/or functional constrains prevent changes in the V3 loop of this CRF and imply that CXCR4 tropism is a stable phenotypic feature of CRF14_BG isolates. What may have determined the evolution of this CRF to a highly pathogenic CXCR4 phenotype is a matter of debate. Escape from neutralizing antibodies targeting the V3 loop may lead to tropism changes both in HIV-1 [665] and HIV-2 [672]. Hence, the convergent evolution of CRF14_BG toward CXCR4 use might have been a direct consequence of successful escape from neutralizing antibodies in infected subjects. Further studies are needed to determine whether CRF14_BG isolates are particularly resistant to neutralization.

Appendix

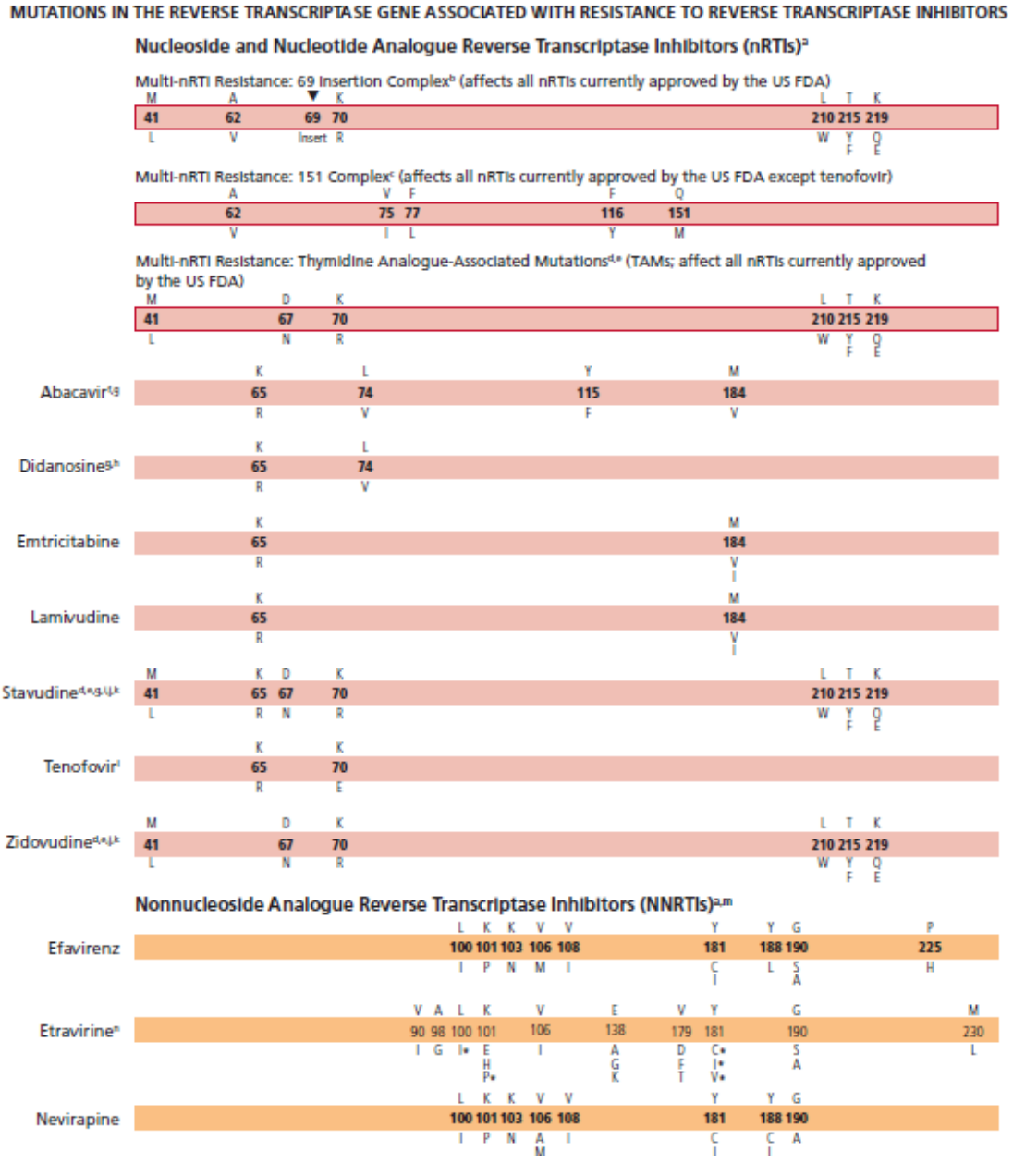

Figure A1 - Drug Resistance Mutations for Antiretroviral Drugs in Clinical Use. Adapted from Johnson *et al.*, *Top HIV Med* 2010 [242].

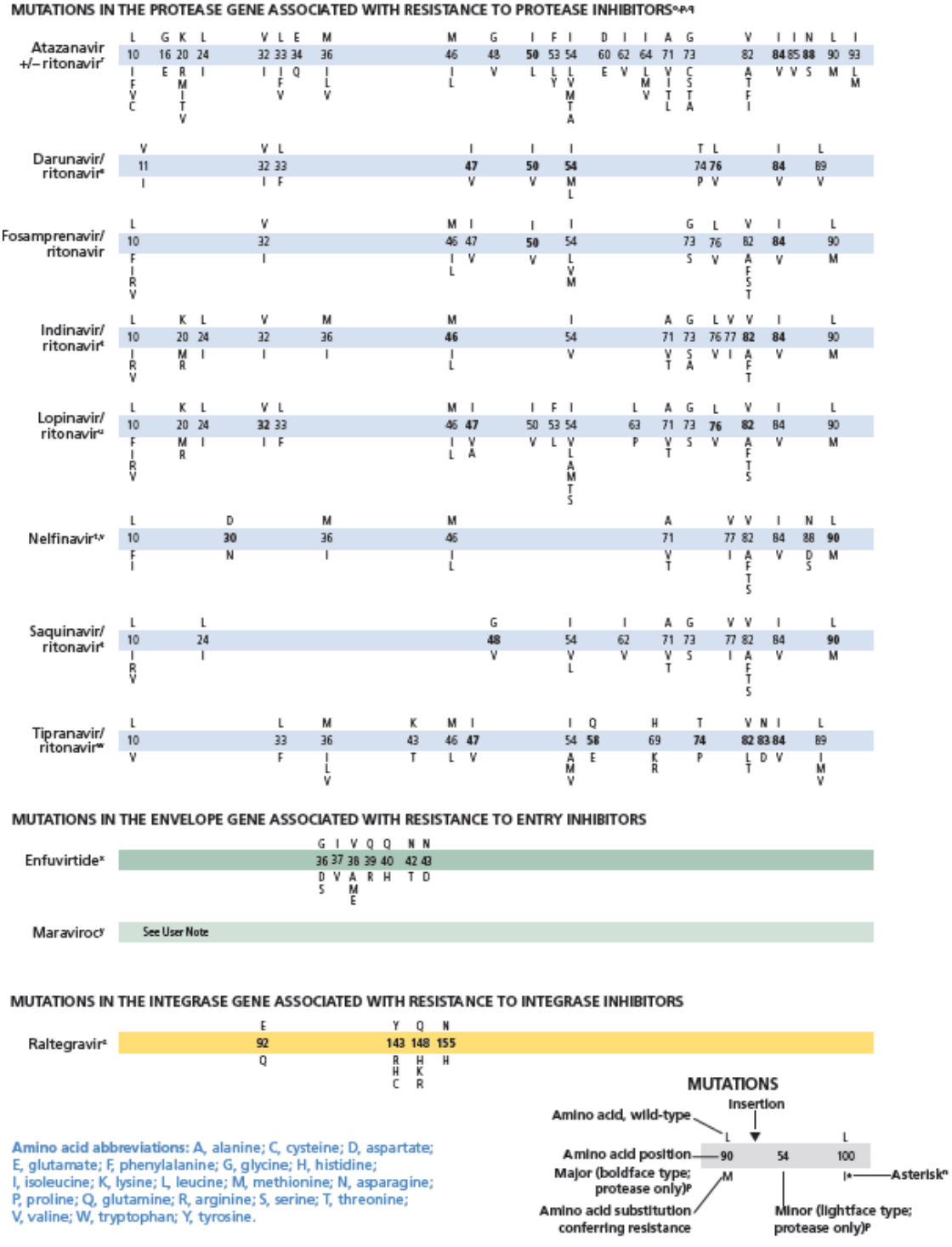

FIGURE A1 *(continued)*

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